RP- HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ISONIAZID AND RIFAMPICIN IN TABLET DOSAGE FORM

Dissertation

Submitted to

The Tamilnadu Dr. M. G. R. Medical University,

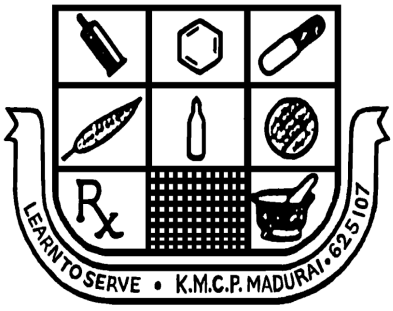
CHENNAI

In partial fulfilment for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS



DEPARTMENT OF PHARMACEUTICAL ANALYSIS

K. M. COLLEGE OF PHARMACY

MELUR ROAD, UTHANGUDI

MADURAI - 625107

APRIL -2014

CERTIFICATE

This is to certify that the dissertation entitled **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ISONIAZID AND RIFAMPICIN IN TABLET DOSAGE FORM"** submitted by **Mr. YELLE SUDHEER KUMAR** (**Reg. No: 261230055**) in partial fulfilment of the degree of Master of Pharmacy in Pharmaceutical analysis under The Tamilnadu Dr.M.G.R Medical University, Chennai, done at **K. M. COLLEGE OF PHARMACY**, MADURAI - 625107, is a bonafide work carried out by him under my guidance and supervision during the academic year April - 2013-2014. The dissertation partially or fully has not been submitted for any other degree or diploma of this university or other universities.

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AN EXPRESION OF GRATITUDE

"With God All Things Are Possible"

Milestones in life are achieved, not by individual efforts but by blessings and guidance of elders, near and dear one of collective wisdom and experience of all those who have shared their views far this project is the product beyond those found within the covers of book. I therefore take this opportunity to express my acknowledgements to all of them.

Let me first thank almighty for giving me life and my parents for educating me and keeping my requirements in priority at all situations. Without their unconditional support and encouragement it would have been impossible to pursue my interest.

It gives me immense pleasure to express my deepest thanks, heartfelt, indebtness and regards to my respected guide, ***Dr. M.SUNDARAPANDIAN.,M.Pharm.,Ph.D.,*** *professor and HOD.,* ***Dept.*** *of Pharmaceutical Analysis, K.M.College of Pharmacy, Madurai, for providing much of the stimuli in the form of suggestions, guidance and encouragements at all stages of my work.*

I owe my great debt of gratitude and heartfelt thanks to ***Prof. M. Nagarajan., M.Pharm.,M.B.A., DMS (IM)., DMS (BM).,****chairman, K.M.College of Pharmacy, Madurai for providing me all the facilities and support for the successful completion of my thesis work.*

I express my deep sincere thanks to ***Dr. S. Venkataraman,M.Pharm., Ph.D.,****Principal, professor and H.O.D, Dept. of Pharmaceutical chemistry, for his timely instigation and immeasurable esteemed help to complete this thesis.*

I express my deep sense of gratitude and profound thankfulness to ***Prof. M.S.Prakash.,M. Pharm.,*** *Dept. of Pharmaceutical Analysis, K.M.College of Pharmacy, and Madurai for his invaluable advice, suggestion and encouragement extended throughout the work.*

I submit sincere thanks and respectful regards ***Mr.T.SRINIVAS REDDY, DIRECTOR, AND MY COLLUGES*** *who provided me all the facilities and his excellent guidance for my project and I am also very thankful to staff of industry for their kind cooperation.*

I extend my thanks to ***M. Shanthi.,*** ***B.A, M.Li.Sc.,*** *Librarian and* ***Mrs. M. Sasi kala.,*** ***D.pharm.,*** *Lab Assistant and* ***Mrs. J.Sridevi.,*** *lab attender and all other non-teaching staff members of our college for their co-operation.*

I cannot forget to express my gratitude and grateful thanks to my classmates ***Ch.Narayana Swamy, Chaitu, Gopi, Sandhya, Sasi ruba and veeraiah*** *who helped me directly and indirectly for the successful completion of my project work.*

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Y. SUDHEER KUMAR

CONTENTS

1. INTRODUCTION

1.1 Introduction to analytical chemistry[1]

The pharmaceutical analysis defined as "the branch of practical chemistry which deals with the resolution, separation, identification, determination and purification of a given sample of a medicine, the detection and estimation of impurities, which may be present in drug substance (or) given sample of medicine".

The substance may be a single compound or a mixture of compounds and may be in the form a tablet, pill, capsule, ampoule, liquid, mixture or an ointment.

The quality control tests involve methods which embrace chemicals, physio – chemical, instrumental, microbiological (or) biological procedures.

The pharmaceutical analysis deals with the subject of determining the composition of material in terms of the elements or compound (drug) present in the system.

Any type of analysis involves two steps

Identification (qualitative)
Estimation (quantitative)

In qualitative analysis, a reaction is performed in such a way as to indicate the formation of a precipitate, a change of a colour, the dissolution of a precipitate complex formation and the evaluation of a gas.

Quantitative analysis is performed ordinarily through five steps. They are sampling, dissolution, precipitation, measurement and calculation.

Analytical Techniques[2, 3,4]

1) Titrimetricmethods

a) Acid – Base titrations
b) Redox titrations
c) Precipitation titrations
d) Non-aqeous titrations
e) Diazotisation titrations

2) Gravimetricmethods

a)Weighing of drug after extraction.
b) Weighing of derivative after separation.
c) Weighing residue after ignition.

3) Spectrophotometric Methods

a) Colourimetric method

b) Ultra violet method

c) Fluorimetric method

d) Flame photometry

e) Atomic absorption spectroscopy.

f) Infrared spectrophotometry

g) Raman spectroscopy

h) X-ray spectroscopy

i) Mass spectroscopy

4) Electro analytical methods

a) Potentiometry

b) Voltametry

c) Amperometry

d) Electrogravimetry

e) Conductometry

f) Polarography

5) Chromatographic methods

a) Thin layer chromatography

b) Paper chromatography

c) Column chromatography

d) Gas chromatography

e) High Performance Liquid Chromatography

6) Hyphenated techniques

a) GC-MS (Gas chromatography – Mass spectrometry)

b) LC-MS (Liquid chromatography – Mass spectrometry)

c) GC-IR (Gas chromatography – Infrared spectroscopy)

d) ICP-MS (Inductively coupled plasma – Mass spectrometry)

7) Miscellaneous methods

a) Thermal analysis

b) Kinetic techniques

c) Enzyme assay

d) Microbiological procedure

e) Biological procedure

DIFFERENT INSTRUMENTAL METHODS WITH BASIC PRINCIPLES

Table No: 1 Different instrumental methods with principles

Sr. N0.	METHOD	BASIC PRINCIPLE

A	ELECTROANALYTICAL METHODS	
1	Potentiometry	Concerned with change in electrical properties of the system measures the change in electrode potential during a chemical reaction of the system
2	Conductometry	Measures the change in electrical conductivity during a chemical reaction
3	Polarography	Measures the current at various applied potential indicating the polarization at indicator electrode
4	Amperometry	Measures the change (or decrease) in current at a fixed potential during addition of titrant
B	SPECTROSCOPIC METHODS	
1	Absorption spectroscopy (Ultraviolet – visible and infrared)	Measures the absorbance or percent transmittance during the interaction of monochromatic radiation (or particular wavelength) by the same
2	Fluorimetry	Measures the intensity of fluorescence caused by emission of electromagnetic radiation due to absorption of UV radiation
3	Flame Photometry	Measures the intensity of emitted light of particular wave length emitted by particular element
4	Turbidimetry	Measures the turbidity of a system by passing light beam in a turbid media

Sr. N0.	METHOD	BASIC PRINCIPLE
5	Nephlometry	Measures the opalescence of the medium by reflection of light by a colloidal solution
6	Atomic Absorption Spectroscopy	Measures the intensity of absorption when atoms absorbs the monochromatic radiation
7	X-Ray Spectroscopy	Measures the position and intensity of spectral lines during emission of X-ray spectrum by atoms under influence of X-rays
8	Refractometry	Measures the refractive index by causing refraction of light by matter
9	Polarimetry	Measures optical reaction by causing the rotation of plane polarized light
C	Mass Spectroscopy	Observe the position and intensity of signals in mass spectrum by causing the ionization of molecules
D	NMR Spectroscopy	Observe the position and intensity lines in NMR spectrum when proton interact with electromagnetic radiation in radio frequency region
E	Thermal Methods	Measures the physical parameters of the system as a function of temperature. It includes thermo gravimetry, derivative gravimetry and differential thermal analysis
F	Radiometric Methods	Measure the radioactivity either present naturally or induced artificially

PHASES IN ANALYTICAL CHEMISTRY[5]

Phase 1: Fast screening phase eg: Immuno assay, gas chromatography and Liquid chromatography.

Phase 2: Identification phase eg: GC – MS.

Phase 3: Qualification phase eg: Spectrophotometer and gas chromatography.

IMPORTANT CONSIDERATIONS IN ANALYTICAL METHODS[6]

The instrument is the most visible and exciting element of the analytical method and it is only one component of the total analysis.

- The analyst should determine the nature of the sample, the end use of the analytical results, the species to be analyzed.
- Quantitative information may include elemental composition, oxidation state, functional groups, major components, minor components, complete identification in the given sample.
- Quantitative data include accuracy, precision and range of expected analyte.
- Methods such as controlling the atmosphere to which the sample is exposed, controlling the temperature of the sample, buffering the pH of sample solutions.

SENSITIVITY AND DETECTION LIMITS [7]

Table No: 2 Sensitivity and detection limits

TECHNIQUES	DETECTION LIMITS	IDENTIFICATION LIMITS
Gas chromatography	10^{-6}-10^{12}	-
Infrared spectrophotometry	10^{-7}	10^{-6}
Ultraviolet spectroscopy	10^{-7}	10^{-6}
N.M.R(time averaged)	10^{-7}	10^{-5}
Mass spectrometry	10^{-6}	10^{-5}
(batch inlet) Mass spectrometry (direct probe)	10^{-12}	10^{-11}

Factors Affecting the Choice of Analytical Methods[8]

- The type of analysis required.
- Problem arising from the nature of the material.

- Possible interference from components of the material other than those of interest.
- The concentration range which needs to be investigated.
- The accuracy required.
- The facilities available.
- The time required for complete analysis.
- Similar type of analysis performed.

1.2 CHROMATOGRAPHY

Chromatography is a technique in which solutes are resolved by differential rates of elution as they pass through a chromatographic column. Chromatography is essentially a

group of techniques for the separation of the compounds of mixtures by their continuous distribution between two phases, one of which moves relative to the other. [4]

History:

Many publications have discussed or detailed the history and development of chromatography. Table No.2 lists the chronological order of the events that are the most notable in the development of the present state of the field. Since the various types of chromatography (liquid, gas, paper, thin-layer, ion exchange, supercritical fluid, and electrophoresis) have many features in common, they must all be considered in development of the field. [9]

Table No: 3 Development of the chromatography

Year	Scientist	Research findings
1834	Runge .F.F	Used unglazed paper and / or pieces of cloth for spot testing dye mixtures and plant extracts
1850	Runge .F.F	Separated salt solutions on paper
1868	Goppelsroeder,F.	Introduced paper strip (capillary analysis) analysis of dyes, hydrocarbons, milk, beer, colloids, drinking and mineral water, plant and animal pigments
1906-1907	Twsett,M.	Separated chloroplast pigment on $CaCO_3$ solid phase and petroleum ether liquid phase
1931	Kuhn,R.et.al	Introduced liquid-solid chromatography for separating egg yolk xanthophylls
1940	Wilson,J.N	Wrote first theoretical paper on chromatography: assumed complete equilibration and linear sorption isotherms, qualitatively defined diffusion, rate of adsorption and isotherm non linearity

1941	Tiselius,A.	Developed liquid chromatography and pointed out frontal analysis, elution analysis and displacement development
1944	Consden, R.,Gordon,A.H., & Martin, A.J.P	Developed paper chromatography
1946	Claesson,S.	Developed liquid-solid chromatography with frontal and displacement development analysis
1951	Cremer,E.	Introduced gas-solid chromatography
1952	James,A.T.,andMartin,A.J.P.	Introduced gas-liquid chromatography
1957	Golay,M	Reported the development of open tubular columns

High Performance Liquid Chromatography

High performance liquid chromatography is the fastest growing analytical technique for the analysis of the drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for an analysis of many drugs in both dosage forms and biological fluids. HPLC was developed in the late 1960s and 1970s. Today it is widely accepted separation technique for both sample analysis and purification in variety of areas. The successful use of liquid chromatography requires the right combination of a variety of operating conditions such as the type of,

- Column packing.
- Mobile phase and its flow rate.
- Column length and diameter.
- Column temperature and sample size.

General uses of HPLC

1. Separation of wide variety of compounds, organic, inorganic and biological compounds, polymers, chiral compounds, thermally liable compounds and small ions to macro molecules.
2. Analysis of impurities.
3. Analysis of both volatile and nonvolatile compounds.

4. Determination of neutral ionic or zwitter ionic molecules.
5. Isolation and purification of compounds.
6. Ultra trace to preparative and process scale separations.
7. Qualitative and quantitative method.[6]

Guide to Liquid Chromatography Mode Selection

Selection of chromatography mode is based upon the analyte polarity, solubility and ionic nature. [7] A guide to liquid chromatography mode selection is represented in fig no: 1

Fig. No: 1 Guide to Liquid Chromatography Mode Selection Reverse Phase HPLC

Reverse phase, aqueous mobile

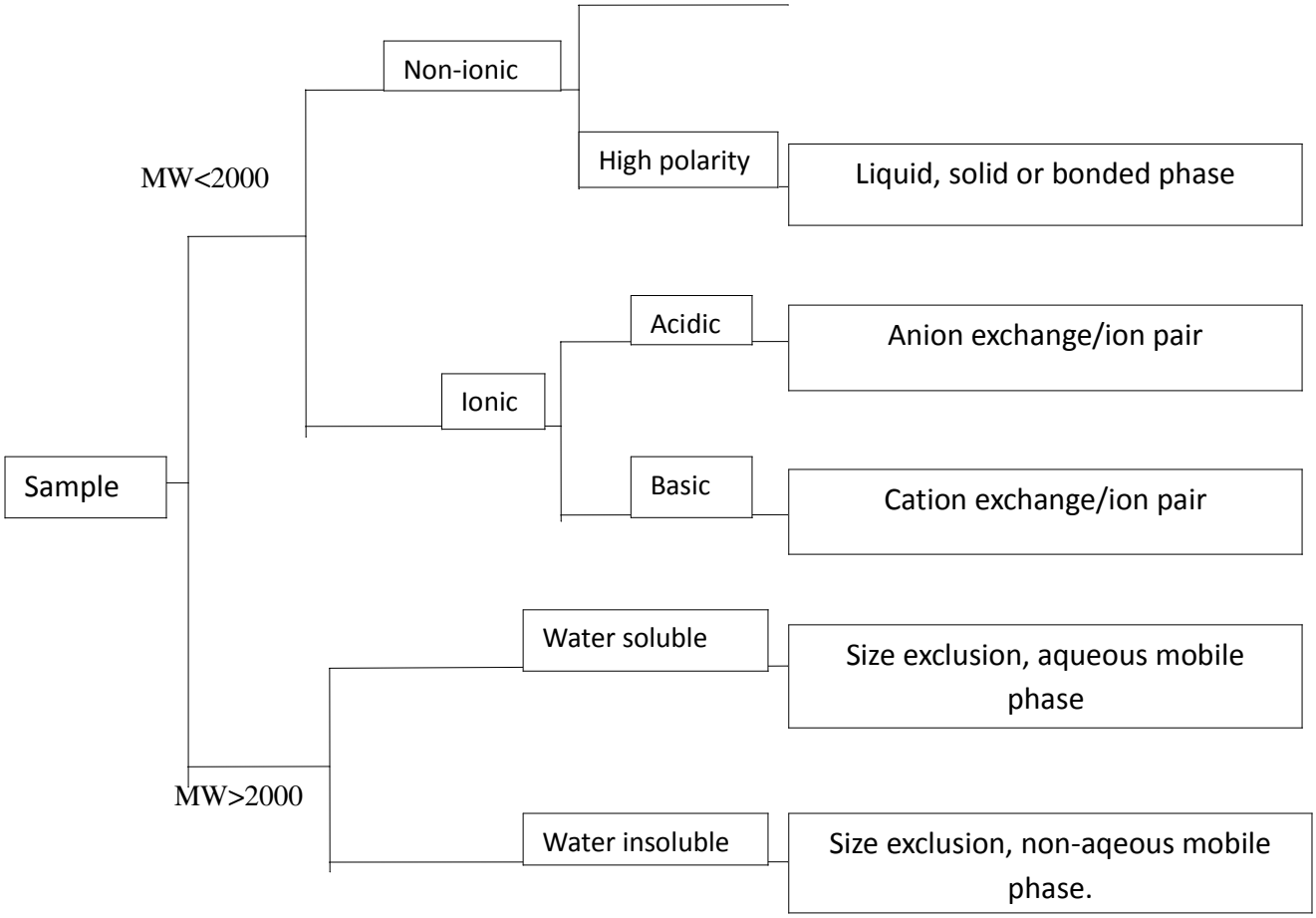
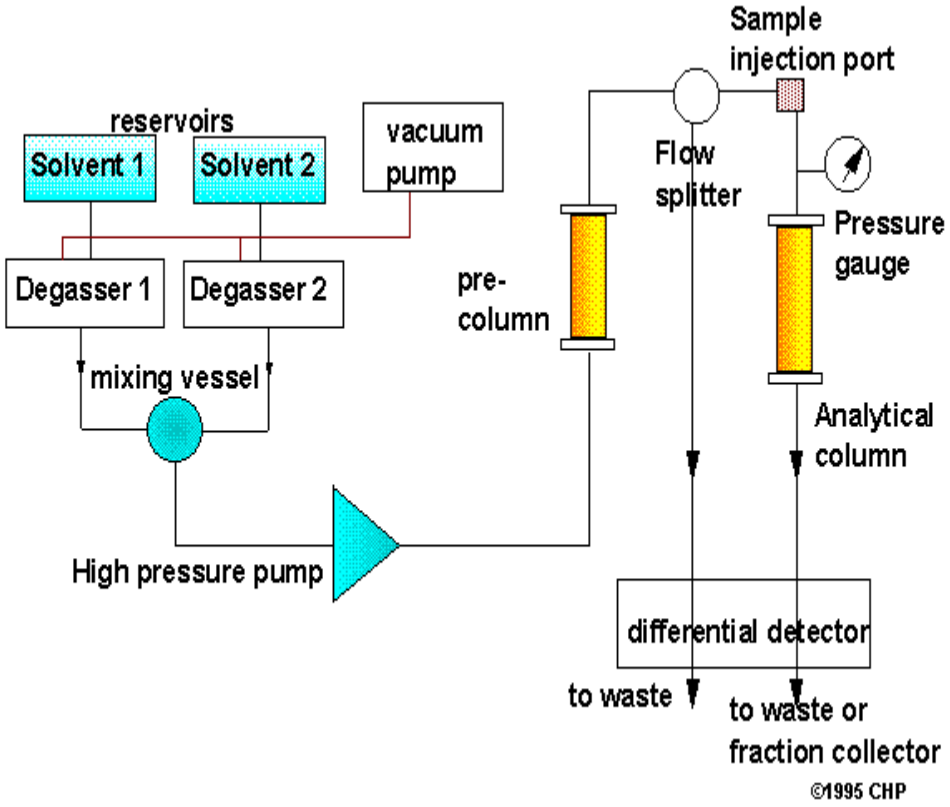


Fig.No: 2 Block diagram showing the components of an HPLC instrument

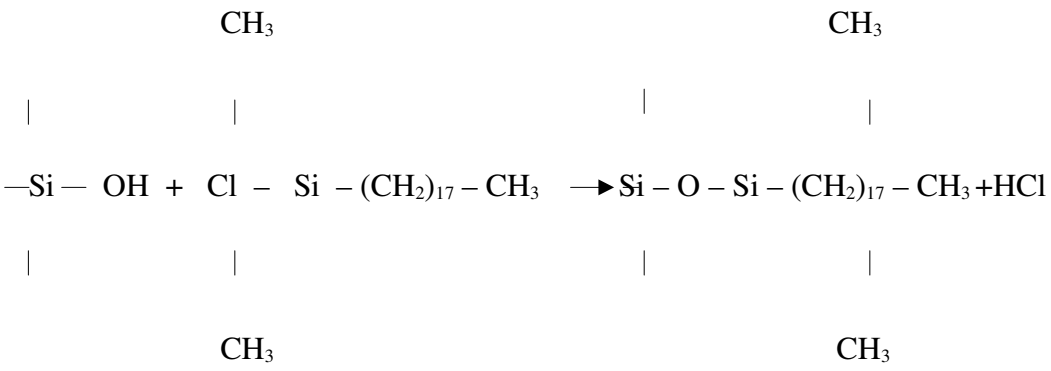

Sample
injection port
reservoirs
Solvent 1
Solvent 2
vacuum
pump
Flow
splitter
Pressure
gauge
Degasser 1
Degasser 2
pre-
column
mixing vessel
Analytical
column
High pressure pump
differential detector
to waste
to waste or
fraction collector
©1995 CHP

F

Reverse phase column packing

The liquid-liquid partition chromatography uses a stationary phase consisting of liquid layer adsorbed to a surface of silica or alumina. In case of bonded phase or reverse phase HPLC uses a stationary phase consisting of an organic moiety chemically bonded to the surface of silica through the surface silanol groups. Since the organic moieties are generally long-chain hydrocarbons, the mobile phases are generally polar. In this mode, the more polar solutes are eluted first while the non-polar compounds are eluted later. The silanol group can react with a chlorosilane group to form the popular (ODS & C_{18}) octaydecylisilane packing.

E.g. Octaydecylisilane (ODS or C_{18}).



For silica based bonded materials, the suitable working pH range is 2 - 8. At pH values of less than 2, the Si-C bond is attached and at the higher pH values, hydrolysis of the siloxane takes place which leads to degradation or destruction of the packing. In most of the applications of RP-HPLC, elution is carried out with highly polar solvents such as methanol, Acetonitrile or tetrahydrofuran in various concentrations. [9a]

Reverse phase mobile phase

The primary constituent of RP-HPLC mobile phase is water. Water miscible solvents such as methanol, ethanol, Acetonitrile, dioxane, tetrahydrofuran and dimethyl formamide are

added to adjust the polarity of the mobile phase. Additionally acids, bases, buffers and/or ionic surfactants are added. The water should be of high quality, either distilled or demineralised water.

The most widely used organic modifiers are methanol, Acetonitrile and tetrahydrofuran. Methanol and Acetonitrile have comparable polarities but Acetonitrile is an aprotic solvent. Ethanol, 1-propanol and 2-propanol are also useful but less polar than methanol. Dioxane, tetrahydrofuran are aprotic solvents that are less polar than Acetonitrile. Reverse phase mobile phases are generally non-flammable due to high water content. Degassing is quite important with reverse phase mobile phases. [9b]

6. DETECTORS[10]

The function of the detector in HPLC is to monitor the mobile phase emerging from the column. The output of the detector is an electrical signal that is proportional to some property of the mobile phase and/or the solutes.

LC detectors are basically of two types.

Bulk property detectors respond to mobile phase bulk property such as refractive index, dielectric constant or density. Solute property detectors respond to some property of solutes, such as UV absorbing, fluorescence, diffusion current, which are not possessed by the mobile phase

Most common HPLC detectors

- UV-Visible absorbance detector (UV-VIS)
- Photo-diode array detector (PDA)
- Fluorescence detector
- Electrochemical (ECD)
- Refractive Index (RI)
- Mass detectors (MS)
- Conductometric detector
- Chiral detector (Polarimetric & circular dichrosim)
- Evaporative Light scattering detector (ELSD)
- Radiochemical detector

Characteristics of ideal detectors

1. Adequate sensitivity.
2. Good stability and reproducibility.
3. A linear response to analyte.
4. A short response time that is independent of flow rate.
5. High reliability.
6. Minimal internal volume in order to zone broadening. [9c]

Liquid chromatographic detectors are of two basic types,

1. Differential or bulk property detector
2. Selective or solute property detector

Differential or bulk property detector

Differential or bulk property detector provides a differential measurement of a bulk property that is possessed by both the analyte and the mobile phase.

e.g., Refractive index, dielectric constant.

Selective or solute property detector

Measure the property of an analyte which is not possessed by the mobile phase.

e.g., UV absorbance, fluorescence.

1.5 ICH GUIDELINES FOR ANALYTICALMETHOD VALIDATION [11]

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated,

- ❖ Before their introduction into routine use
- ❖ Whenever the conditions change for which the method has been validated, e.g., instruments with different characteristics.
- ❖ Whenever the method is changed and the change is outside the original scope of the method. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures.

REASONS FOR VALIDATION

1. Enables scientists to communicate scientifically and effectively on technical matters.
2. Setting standards of evaluation procedures for checking complaints and taking remedial measures
3. Retrospective validation is useful for trend comparison of results compliance to cGMP/GLP.
4. Closer interaction with pharmacopoeia harmonization particularly in respect of impurities determination and their limits.
5. For taking appropriate action in case of non – compliance.

6. To provide high degree of confidence that the same level of quality is consistently built into each unit of finished product from batch to batch.

As quality control process is not static, some form of validation / Verification should continue till the validated process is in use.

The parameters as defined by the ICH and by other organizations

- Specificity
- Selectivity
- Precision
  - Repeatability
  - Intermediate precision
  - reproducibility
- Accuracy
- Linearity
- Range
- Limit of detection
- Limit of quantification
- Robustness
- Ruggedness

A) Specificity and selectivity

i. Selectivityistheabilitytomeasureaccuratelyandspecificallytheanalyteinthepresence of components that may be expected to be present in the sample matrix.
ii. Specificity for an assay ensures that the signal measured comes from the substance of interest and that there is no interference from excipients and/or degradation products and/or impurities.
iii. Determination of this can be carried out by assessing the peak identity and purity.
iv. Diodearray detectors can facilitate the development and validation of HPLC assays. Spectra] data obtained from diode array detectors, effectively supplement the retention time data for peak identification, also spectral manipulation often provides information about the peak purity. The table below lists several of the techniques available for assessing peak identity and purity.
v. The purity index is a measure of the peak's relative purity, measured using a full comparis on of spectral data for the leading and training edge of the peak. A value of 1.5 is commonly accepted to indicate a pure peak.

But>1.5wouldindicatethepresence of an impurity.

B) Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed at the variance, standard deviation or coefficient of variation of a series of measurements.

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Precision is a measure of the reproducibility of the *whole* analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to
Obtain statistically valid results (i.e.between6-10). The precision is then expressed as the relative standard deviation

$$\%RSD = \frac{STDdevx100\%}{Mean}$$

Repeatability

Express the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra - assay precision. It should be assessed using a minimum of nine determinations covering the specified range for the procedure (e.g. three concentration/three replicates each) or a minimum of determinations at 100% of the test concentration.

Intermediate precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc.

Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance inclusion of procedures in Pharmacopoeias.

C) Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or on an accepted reference value and the value found.

Assay

- Assay of Active Substance
- Assay of Medicinal products

Several methods are available to determine the accuracy.

a) Application of an analytical procedure to an analyte of known purity (e.g. reference material).
b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure)
c) Application of the analytical procedure to synthetic mixtures of the product components to which known quantities of the substance to be analyzed have been added.

Impurity (Quantification)

Accuracy should be assessed on sample (substance /products) spiked with known amounts of impurities. It should be clear how the individual or total impurities are to be determined.

E.g. Weight / Weight or area percent.

D) Linearity and range

Linearity of an analytical procedure is its ability (with in a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods. For example, calculation of a regression line by the method of least square. Therefore data from regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

- Range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample including these concentrations for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The following minimum specified ranges should be considered

- For the assay of an active substance or a finished product normally from 80-120 percent of the test concentration.
- For the content uniformity, covering a minimum of 70-130 percent of the test concentration.
- For dissolution testing, 20% over the specified range (e.g.), If the specifications for a controlled release product cover a region from 20% after 1 hour, upto 90% after 24 hours, the validated range would be 0-110% of label claim.
- For the determination of an impurity, the reporting level of an impurity to 120% of the specifications.

E) Limit of Detection

The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing that minimum level at which the analyte can reliably detected.

i. Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may be used with instrumental methods.

ii. Based on signal to noise ratio

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio 3 or 2:1is generally considered acceptable for estimating the detection limit.

iii. Based on the standard deviation of the response and the slope

The detection limit (DL) may be expressed as

$$D L = 3.3/S$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope, S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways.

a. Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

b. Based on the calibration curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercept of regression lines may be used as the standard deviation

F) Limit of Quantification

The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision

i. Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

ii. Based on Signal- to-Noise ratio

Determination of the Signal-to-Noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical Signal- to-Noise ratio is 10:1.

iii. Based on the Standard Deviation of the Response and the slope

The quantification limit (Q L) may be expressed as

$$QL = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimation may be carried out in a variety of ways including,

a. Based on standard deviation of the blank

Measurement of the magnitude of analytical background response is performed by an appropriate number of blank samples and calculating the standard deviation of these responses.

b. Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation

G) Robustness

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

Examples of typical variations are

- Stability of analytical solutions.
- Extraction time.

In case of liquid chromatography, examples of typical are

- Influence of variation of pH in a mobile phase,
- Influence of variations in mobile phase composition,
- Different columns (different lots and / or suppliers),
- Temperature,
- Flow rate.

In the case of gas-chromatography, examples of typical variations are

- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

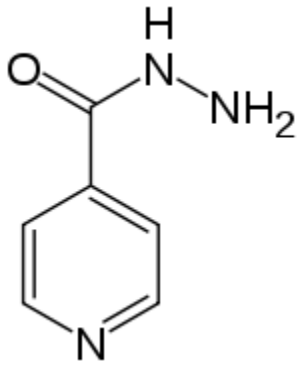
F) Ruggedness

The United States pharmacopoeia (USP) define ruggedness as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different labs, different analysis, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from analyst to analyst.

2. DRUG PROFILE

2.1 Isoniazid[12]

Structure



Nomenclature : Isonicotinohydrazide

Molecular Formula : $C_6H_7N_3O$

Molecular Weight : 137.1 g/mol

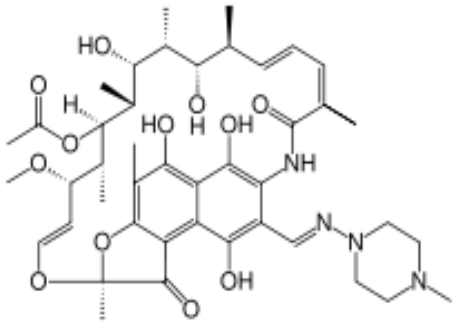
Appearance : A white or almost white, crystalline powder or Colourless crystals

Solubility : freely soluble in water, sparingly soluble in Alcohol

Category : Anti–Tuberculosis

2.2 Rifampicin[13]

Structure



Nomenclature **:** (7*S*,9*E*,11*S*,12*R*,13*S*,14*R*,15*R*,16*R*,17*S*,18*S*,19*E*,21*Z*)-2,15,17,27,29-pentahydroxy-11-methoxy-3,7,12,14,16,18,22-heptamethyl-26-{(E)-[(4-methylpiperazin-1-yl)imino]methyl}-6,23-dioxo-8,30-dioxa-24-azatetracyclo[23.3.1.1^{4,7}.0^{5,28}]triaconta-1(28),2,4,9,19,21,25(29),26-octaen-13-yl acetate

Molecular Formula **:** $C_{43}H_{58}N_4O_{12}$

Molecular Weight **:** 823 g/mol

Appearance **:** Reddish brown colour, brownish red crystalline Powder

Solubility **:** Sparingly soluble in water, freely soluble in Methanol, sparingly soluble in acetone, ethanol(96%).

Category **:** Anti–Tuberculosis

3. REVIEW OF LITERATURE

Shah Y, *et al.,*[14] developed a liquid chromatographic procedure for the analysis of Rifampicin (RIF) and Isoniazid (ISN) in pharmaceutical dosage forms utilizing reverse phase chromatography. Isolation of analytes was carried out under isocratic condition with an octaydecylisilane column and an aqueous mobile phase containing methanol (75%) and 0.02M disodium hydrogen orthophosphate (25%) with pH 4.5 was adjusted with orthophosphoric acid. The detection was done at 254mm. The method was unique in analysing Rifampicin precisely in liquid combination with Isoniazid particularly in liquid formulation. The method was specific and could distinctly isolate the degradation product in suspension.

Glass BD, *et al.,*[15]developed a stability – indicating HPLC method for the simultaneously determination of Rifampicin, Isoniazid and pyrazinamide in a fixed dose combination. The best separation and reasonable short retention times were produced on the micro bondapak C_{18}, 4.6x250 mm column, 10 microns/125 using ACN:tBAH (42.5:57.5, v/v) (0.0002M) as the mobile phase with optimized rate at final pH.

Caller E, *et al.,*[16] developed and validated simple and accurate liquid chromatographic method for estimation of Isoniazid (ISN), pyrazinamide (PZA) and Rifampicin (RIF) in combined dosage forms. Drugs were chromatographed on a reverse phase C_{18} column using a mobile phase gradient and monitored at the corresponding maximum of each compounds. The method was linear (r^2>0.999), précised (RSD%:0.50 % for ISN, 0.12% for PYR and 0.98% for RIF), accurate (overall average recovery yields 98.55% for ISN, 98.51 for PYR and 98.56 for RIF) and selective.

Mohan B, *et al.,*[17] reported USP gradient HPLC method for quantitative determination of Rifampicin, Isoniazid and pyrazinamide in fixed dose combination (FDC) formulations to determine its ability to resolve major degradation products of Rifampicin, viz 3-formylrifamycin SV, Rifampicin N-oxide, 25-desacetyl Rifampicin, Rifampicin quinine andisonicotinylhydrazone an interaction product of 3-formylrifamycin and Isoniazid. The first observation was that the requirements of theoretical plates listed in the given method were met for Rifampicin, but not for Isoniazid and pyrazinamide even on columns of different makes. The resolving power of the method was also dependent upon make of the column. On two of the three columns of the three tested, it was able to resolve most degradation products, except Rifampicin N-oxide and 25 – desacetylRifampicin, which were overlapping. The method was modified and an overall satisfactory resolution for all components was obtained by changing the buffer organic modifier ratio of solution B in the gradient from 45:55 to 55:45 and decreasing the flow rate from 1.5 to 1.0 ml/min, keeping all other conditions constant.

Panchangul R, *et al.,* [18] developed a reversed phase HPLC method for the simultaneous estimation of Rifampicin and its main metabolite in plasma and urine in presence of pyrazinamide and Isoniazid. The assay involved simple liquid extraction of drug, metabolite and internal standard (rifapentine) from biological specimens and their subsequent separation on aC_{18} reversed phase column and single wave length UV detection. In plasma as well as in urine samples, all the three compounds of interest eluted within 17 min, using methanol: sodium phosphate buffer (pH 5.2:0.01mm) (65.35, v/v) as mobile phase under isocratic conditions. It was established that Isoniazid, pyrazinamide and ascorbic acid (added to prevent oxidative degradation of analytes) did not interfere with the analyte peaks. Recoveries (extraction efficiency) for drug were greater than 90 % in both plasma and urine, whereas for metabolite the values were found to be 79 and 86 % in plasma and urine respectively. The plasma and urine methods were précised and accurate for both the analytes. The method developed was proved to be suitable for simultaneous estimation of Rifampicin and desacetylRifampicin in plasma and urine samples.

Smith PJ, *et al.,*[19]developed simple and sensitive high performance liquid chromatographic (HPLC) method suitable for assaying RIF, INH and PZA in bio availability studies. RIF, desacetylRifampicin (DRIF), INH and PZA were extracted simultaneously from plasma using a solid phase extraction column. RIF and DRIF were quantitated by using an 80% Acetonitrile 10.1% trifluoroacetic acid (TFA) as mobile phase and C_8 reversed phase column .INH and PZA were also quantitated by using C_8 reversed phase column, but mobile phase was 3% Acetonitrile 10.6% TFA. Mean recovery of RIF, DRIF and PZA from plasma was well over90% and over 70% for INH. Calibration graphs were linear for all the drugs in their therapeutic range. Correlation coefficients were all above 0.9995.

Khuhawar MY, et *al.*, [20] developed liquid chromatographic method for determination of Isoniazid, Pyrazinamide andRifampicin from pharmaceutical preparations and blood. Isoniazid (ISN), pyrazinamide (PZA) and Rifampicin (RIF) were separated on YMC ODS column. ISN was derivated with 2 – fluorence – carboxaldehyde (FCA). The separation was achieved using ethanol – chloroform – Acetonitrile water by isocratic elution and detected at 337mm. The detection limits were 0.11 ng, 0.2 ng and 113 ng/ injection (5 μ l) for ISN, PZA and RIF, respectively. The method of analysis was applied to the pharmaceutical preparations and in the blood samples of the patients suffering from tuberculosis after undergoing chemotherapy with ISN, PZA and RIF. The amounts quantitated in blood showed 0/97 to 1.58 µg/ml ISN, 3.44 to 409 µg/ml Pz and 1.98 to 3.5 µg/ml RIF with coefficient of variations 0.8 – 1.8%, 0.9 – 1.3% and 0.8 – 2% respectively.

Ali. J, *et al.,* [21] developed simple, selective, and précised and stability indicating high performance thin layer chromatographic (HPILC) method. The compounds were separated an aluminium backed silica gel 60 F254 plates with r-hexane: 2-propanal: acetone: ammonia: formic acid, 3:3.8:2.8:0.3:0.1 (v/v) as mobile phase. The correlation coefficients were 0.994 and 0.997 for Isoniazid and Rifampicin respectively. The values of slope and intercept of the calibration plots were 3.755 +/_ 0.22 and 3099.1+/_ 51.21 respectively, for Isoniazid and 4.0957

+/_ 0.25and 3567.6 +/- 61.11 respectively. For Rifampicin. The method was validated for precision, recovery and robustness. The limits of detection and quantification were 2010.51 and 60+/- 1.05 ng respectively, for Isoniazid and 2510.63 and 75+/- 1.12ng respectively. In stability tests the drugs were susceptible to acid and basic hydrolysis, oxidation and photo degradation.

Kakde R, *et al.,* [22] developed three spectrophotometric methods for the determination of Rifampicin and Isoniazid in pharmaceutical preparations. First method was based on the determination of graphical absorbance ratio at two selected wavelengths. In the second method derivative spectroscope was used to eliminate spectral interference and the third method was based on the additivity of absorbance. All the three methods were found to be simple, rapid, and accurate and could be adopted in routine analysis of drug in formulations.

Balbo MS,*et al.,* [23] developed sensitive and reproducible stir bar sorptive extraction and HPLC – UV detection (SBSE/HPL - UV) method for therapeutic drug monitoring of Rifampicin in plasma samples. Separation was obtained using reverse phase C_8 column with UV detection (254mm). The mobile phase consisted of methanol: 0.25N sodium acetate buffer, pH 5.0 (58:42, v/v). The SBSE/HPLC - UV method was linear over a working range of 0.125 – 50.0 µg ml^{-1}. The intra assay and inter assay precision and accuracy was studied at three concentrations (1.25, 6.25 and 25.0 µg ml-1). The intra assay coefficients of variation (CVS) for all compounds were less than 10%. Limit of quantification was 0.125 µg ml-1. Stability studies showed that Rifampicin was stable in plasma for 12 hour after thawing and the samples were also stable for 24 hour after preparation.

Satavi A, *et al.,* [24] developed a simple, rapid, sensitive and accurate indirect spectrophotometric method for the micro determination of Isoniazid (INH) in pure form and pharmaceutical formulations. The procedure was based on the reaction of copper (II) with Isoniazid in the presence of neocuproine (NC). In the presence of neocuproine, copper (II) was reduced easily by Isoniazid to a cu (I) neocuproine complex, which showed an absorption Maximum at 454nm. By

measuring the absorbance of the complex at this wavelength, Isoniazid could be determined. This method was applied to the determination of Isoniazid in pharmaceutical formulation and enabled the determination of the Isoniazid in microgram quantities. The results obtained for the assay of pharmaceutical preparative compared well with those obtained by the official method and demonstrated good accuracy and precision.

Rui L, *et al.*, [25] developed fluorimetric method for the determination of Isoniazid by oxidation with cerium (IV) in a multicommutated flow system. A multicommutated flow system for the indirect fluorimetric determination of Isoniazid procedure was based on the oxidation of Isoniazid by cerium (IV) and monitoring of the fluorescence intensity of the formed cerium (III). Linear calibration plots up to 1.37 Mg Ml-1, with a relative standard deviation of less than 1.6% (n=12) and a sample through put of about 50 samples per hour, were obtained. The lower detection limit was 34.3ng Ml-1. The methodology was evaluated in the determination of Isoniazid in pharmaceutical preparations and the effect of potential interferences was investigated.

4. AIM AND PLAN OF WORK

Aim and scope

Isoniazid and Rifampicin is official in I.P, B.P, and U.S.P. From the literature survey, it was found that there were only few RP-HPLC methods reported for the estimation of Isoniazid and Rifampicin in pharmaceutical dosage forms.

Hence, the aim of present work is to develop RP-HPLC method for estimation of Isoniazid and Rifampicin from the tablet dosage form and to validate the developed RP-HPLC method by validation parameters as per ICH guidelines.

Plan of present work

- Method development (By reverse phase HPLC).
- Validation of the developed method (By using following parameters).
    a. System suitability studies
    b. Accuracy
    c. Precision
    d. Linearity
    e. Specificity
    f. Robustness
    g. Limit of detection
    h. Limit of quantification

5. MATERIALS AND INSTRUMENTS USED

S.No.	NAME	MODEL	MANFACTURER/SUPPLIER
1.	Weighing balance	AUM220D	Shimadzu
2.	Sonicator	Sonorex	Sonorex dg 10p
3.	pH Meter	9087	ELICO pH METER
4.	HPLC-UV,PDA	Waters e 2116	Waters
5.	Colum	Zodiac C_{18}	Zodiac

a) Apparatus / Instruments used:

b) Active Ingredients used:

S.No.	NAME	SPECIFICATION
1.	Isoniazid	As Reference standard
2.	Rifampicin	As Reference standard

c) Chemicals used:

S.NO	NAME	MODEL	MANFACTURER/SUPPLIER
	Pottasiumdihydrogen		

1.	orthophosphate	HPLC	Rankem Chemicals
2.	Methanol	HPLC	Rankem Chemicals
3.	Phosphoric acid	HPLC	Merck Chemicals
4.	Milli-Q Water	HPLC	In House production

6. RP-HPLC METHOD DEVELOPMENT

In case of analytical method development and for drugs analysts should decide whether the given analytical method is suitable for the assay of the drug. The method development of new improved method usually trailers existing approaches and instrumentation to the current analyte, as well as to the final needs or requirements of the method.

In the development stage, decision regarding choice of column, mobile phase, detectors and method of quantitation must be addressed. In this way, development considers all the parameters pertaining to any methods.

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(1) Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample andchemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography.

From literature survey, it was found that different C_{18}columns could be appropriately used for the separation ofIsoniazid and Rifampicin.

(2) Selection of wavelength

The sensitivity of the HPLC depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for both drugs to be detected. The wavelength for measurement was selected as 263 nm from the absorption spectrum.

(3) Selection of mobile phase

The mobile phase was selected andchromatograms were recorded, trails were done on Isoniazid and Rifampicin.

ASSAY METHOD DEVELOPMENT

The objective of this experiment was to optimize the assay method for simultaneous estimation of Isoniazid and Rifampicinbased on the literature survey made and the methods given in pharmacopoeia. Trials done for optimization were as follows:

Trials

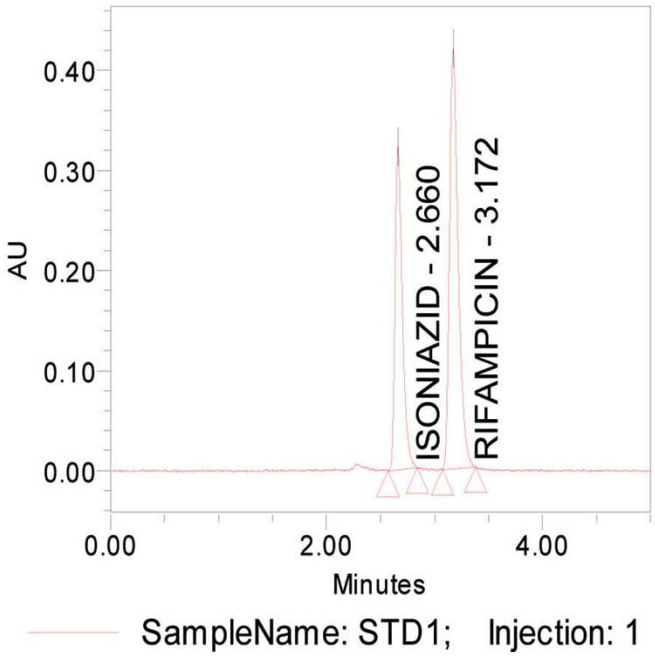
Buffer preparation: 17.418 gm of Potassium Hydrogen Orthophosphate was mixed with 1000 ml of Mille-Q water and shaked for 15min and degassed.

Trial 1:

Mobile phase A	: Potassium Hydrogen Orthophosphate
Mobile phase B	: Methanol

Chromatographic conditions

Column	: Agilent Zorbax Sb-C18, (4.6 × 250 mm, 5 µ)
Detector wavelength	: 263 and 274 nm
Column temperature	: 30 ^{0}C
Injection Volume	: 10 µl
Flow rate	: 1.0 ml/min
Runtime	: 15 min



Observation: Tailing was not satisfactory for both Rifampcin and Isoniazidand the retention time of Rifampicin and Isoniazid were found to be 3.17 and 2.6 minutes respectively.

Trial 2: (OPTIMIZED METHOD)

Buffer Preparation: 17.418 gm of Potassium Hydrogen Orthophosphate was mixed with 1000 ml of Mille-Q water and shaked for 15min and degassed.

Mobile phase A : Potassium Hydrogen Orthophosphate

Mobile phase B : Methanol

Chromatographic Condition:

Column : AgilentZorbax Sb-C18, (4.6 × 250 mm, 5 µ)

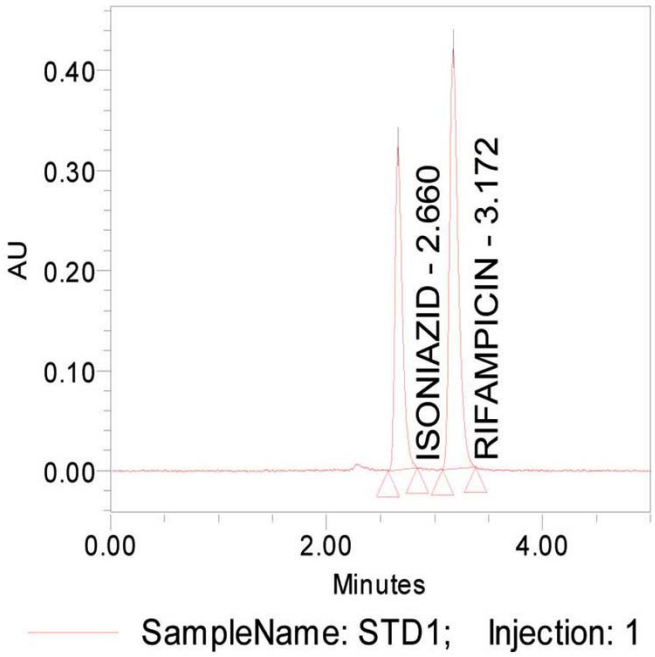
Column temperature : 30^0C

Inj. Volume : 10 µl

Flow rate : 1.0 ml/min

λ_{max} : 263 nm

Run time : 15 min



Observation: Tailing was satisfactory for both Rifampcin and Isoniazid and the retention time of Rifampicin and Isoniazid were found to be 3.17 and 2.6 minutes respectively.

Sample preparation

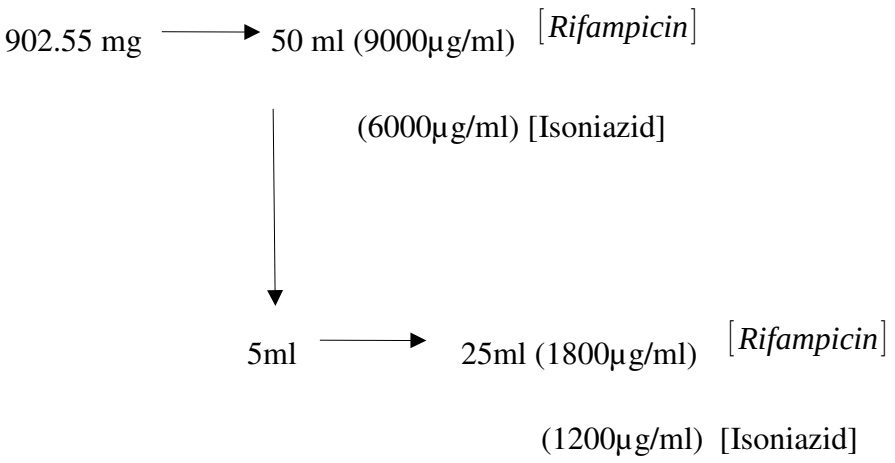
Preparation of sample solution of Isoniazid and Rifampicin for trials:

10 tablets were weighed accurately and finely powdered. Tablet powder of902.55 mg equivalent to 450 mg of Rifampicin and 300 mg of Isoniazid was weighed and transferred into a 50 ml standard volumetric flask. After this 25 ml of HPLC Water (diluent) was added and sonicated for 30 minutes with intermittent shaking and cooled to room temperature. Volume was made with HPLC Water (diluent) and mixed well.

5ml of stock was pipetted out in to a 25ml standard volumetric flask and finally volume was made up with 25ml HPLC water (diluents). This solution was referred as Rifampicin and Isoniazid sample solution that contained 1800 µg of Rifampicin and 1200 µg of Isoniazid per ml respectively.

The scheme of dilution was presented as follows

Dilution chart:



Standard solution preparation:

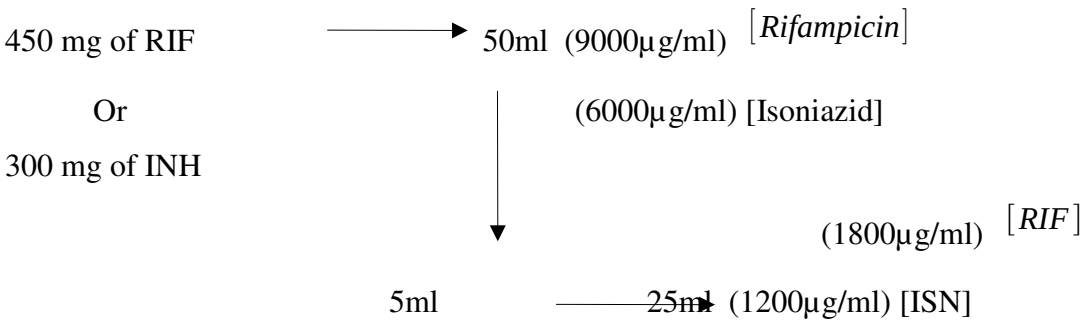
450 mg of Rifampicin (RIF) and 300 mg of Isoniazid (ISN) was accurately weighed and transferred into a 50 ml standard volumetric flask. After this 5 ml of HPLC water was added and sonicated for 30 minutes with intermittent shaking and cooled to room temperature. Volume was made with diluent and mixed well.

5ml of stock was pipetted out in to a 25ml standard volumetric flask and finally volume was made up with 25ml HPLC water (diluents). This solution was referred as Rifampicin and Isoniazid sample solution that contained 1800 µg of Rifampicin and 1200 µg of Isoniazid per ml respectively.

The scheme of standard dilution was presented as follows

Dilution chart:

Standard preparation



7. METHOD VALIDATION

VALIDATION

According to ICH guidelines method validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".Such validated analytical method for qualitative and quantitative testing of the drug molecule assume greater importance when they are employed to generate quality and safety compliance data during development, pre-formulation studies and post approval of drug products.

The ICH of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics

Parameters Used for Assay Validation

The validation of the assay procedure was carried out using the following parameters.

1) Parameters:

1.1 System suitability

1.2 Specificity

1.3 Method Precision

1.4 Linearity & range

1.5 Accuracy / Recovery studies

1.6 Robustness

PREPARATION OF STANDARD AND SAMPLE SOLUTION:

STANDARD PREPARATION:

Buffer Preparation: 17.418 gm of Potassium Hydrogen Orthophosphate was mixed with 1000 ml of Mille-Q water and shaked for 15min and degassed.

Mobile phase A : Potassium Hydrogen Orthophosphate

Mobile phase B : Methanol

Chromatographic Condition:

Column : Agilent Zorbax Sb-C18, (4.6 × 250 mm, 5 µ)
Column temperature : 30^0C
Inj. Volume : 10 µl

Flow rate : 1.0 ml/min
λ_{max} : 263 nm
Runtime : 15

Sample preparation

Preparation of sample solution of Isoniazid and Rifampicin for trials:

10 tablets were weighed accurately and finely powdered. Tablet powder of 902.55 mg equivalent to 450 mg of Rifampicin and 300 mg of Isoniazid was weighed and transferred into a 50 ml standard volumetric flask. After this 25 ml of HPLC Water (diluent) was added and sonicated for 30 minutes with intermittent shaking and cooled to room temperature. Volume was made with HPLC Water (diluent) and mixed well.

5ml of stock waspipetted out in to a 25ml standard volumetric flask and finally volume was made up with 25ml HPLC water (diluents). This solution was referred as Rifampicin and Isoniazid sample solution that contained 1800 µg of Rifampicin and 1200 µg of Isoniazid per ml respectively.

The scheme of dilution was presented as follows

Dilution chart:

902.55 mg ⟶ 50 ml (9000µg/ml) [*Rifampicin*]

(6000µg/ml) [Isoniazid]

↓

5ml ⟶ 25ml (1800µg/ml) [*Rifampicin*]

(1200µg/ml) [Isoniazid]

Standard preparation

Standard solution preparation:

450 mg of Rifampicin (RIF) and 300 mg of Isoniazid (ISN) was accurately weighed and transferred into a 50 ml standard volumetric flask. After this 5 ml of HPLC water was added and sonicated for 30 minutes with intermittent shaking and cooled to room temperature. Volume was made with diluent and mixed well.

5ml of stock waspipetted out in to a 25ml standard volumetric flask and finally volume was made up with 25ml HPLC water (diluents). This solution was referred as Rifampicin and Isoniazid sample solution that contained 1800 µg of Rifampicin and 1200 µg of Isoniazid per ml respectively.

The scheme of standard dilution was presented as follows

Dilution chart:

450 mg of RIF ⟶ 50ml (9000µg/ml) [*Rifampicin*]

Or (6000µg/ml) [Isoniazid]

300 mg of INH

↓

(1800µg/ml) [*RIF*]

5 ⟶ 25ml (1200µg/ml) [ISN]

SYSTEM SUITABILITY

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Before performing any validation experiment, HPLC method and the procedure should be capable of providing data of acceptable quality. These tests are to verify that the resolution and repeatability of the system are adequate for the analysis to be performed. It is based on the concept that equipment, electronics, analytical operations and sample constitute an integral system that can be evaluated as a whole. System suitability parameters and recommendations were shown in the table no.3

- **Table no. 3 System suitability parameters and recommendations**

S.No	Parameters	Recommendations
1	Theoretical plates (N)	>2000
2	Tailing factor (T)	≤ 2
3	Resolution (Rs)	> 2 between peak of interest and the closest eluting potential interference
4	Repeatability	RSD ≤ 1% for N ≥5 is desirable
5	Capacity factor (k^1)	> 2.0
6	Relative retention	Not essential as long as the resolution is stated

Procedure:

- A standard solution was prepared by using Isoniazid and Rifampicin working standards as per test method and was injected six times into the HPLC system.
- The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from ten replicate injections for Isoniazid and Rifampicin retention times and peak areas. Resulted chromatogram was shown in the chromatogram fig. no.3.

Fig no.3Chromatogram of standard 1

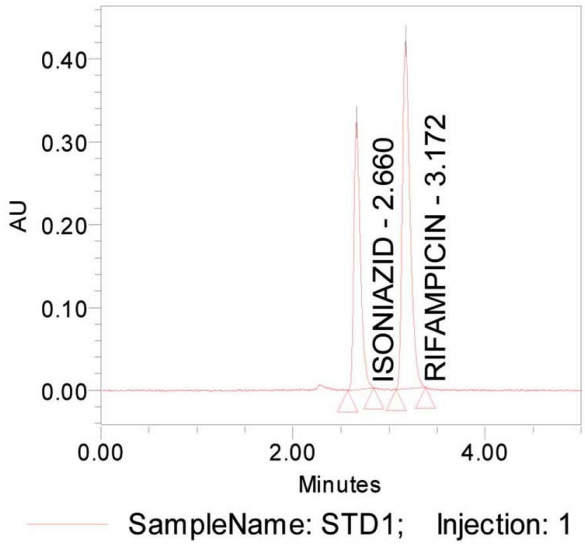


Table no.4 Data for system suitability of ISONIAZID

Name: ISONIAZID

	SampleNam	Inj	Name	RT	Area	USPResolution	USPTailing	USPPlateCoun
1	STD1	1	ISONIAZI	2.660	1518803		1.469	7755
Mean					1518803			
%RSD								

Table no.5 Data for system suitability of RIFAMPICIN

Name: RIFAMPICIN

	SampleNam	Inj	Name	RT	Area	USPResolutio	USPTailing	USPPlateCount
1	STD1	1	RIFAMPICI	3.172	2348101		1.412	7613
Mean					2348101			
%RSD								

Fig no.3Chromatogram of standard 1

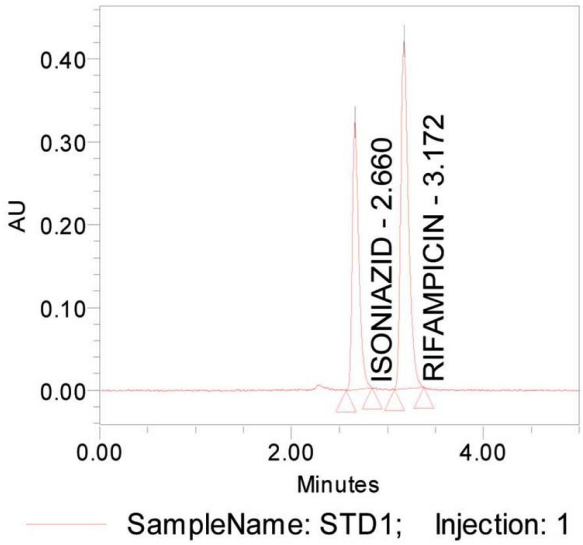


Table no.4 Data for system suitability of ISONIAZID

Name: ISONIAZID

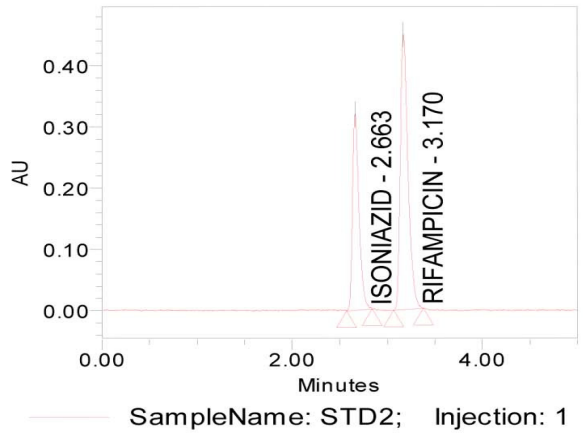
	SampleName	Inj	Name	RT	Area	USPResolution	USPTailing	USPPlateCount
1	STD1	1	ISONIAZID	2.660	1518803		1.469	7755
Mean					1518803			
%RSD								

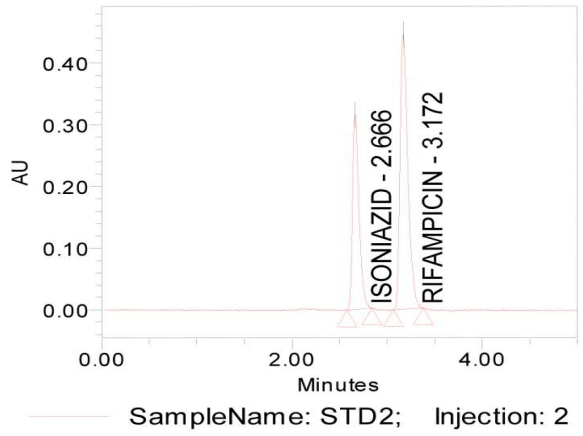
Table no.5 Data for system suitability of RIFAMPICIN

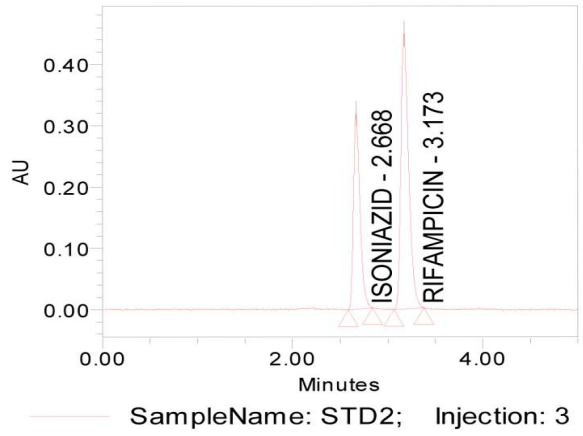
Name: RIFAMPICIN

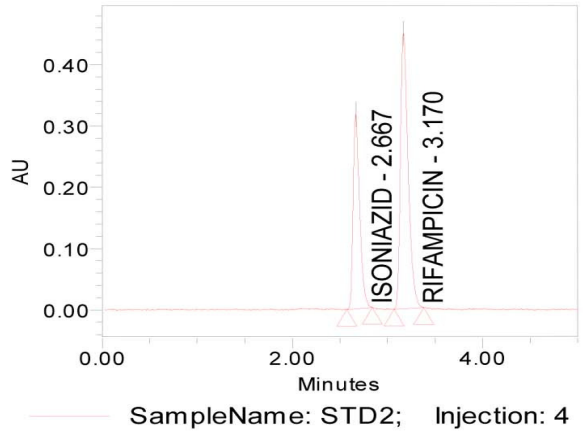
	SampleName	Inj	Name	RT	Area	USPResolution	USPTailing	USPPlateCount
1	STD1	1	RIFAMPICIN	3.172	2348101		1.412	7613
Mean					2348101			
%RSD								

Fig no.4Chromatograms of standard 2









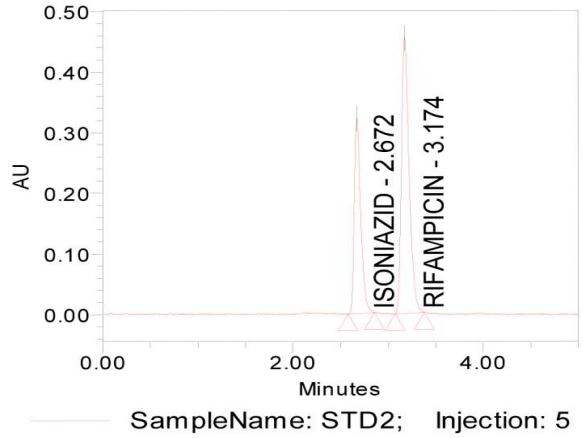


Table no.6 Results of system suitability (ISONIAZID)

Name: ISONIAZID

	Sample N	Inj	Name	RT	Area	USPResolutio	USP Tailing	USPPlateCount
1	STD2	1	ISONIAZI	2.663	151632		1.436	7479
2	STD2	2	ISONIAZI	2.666	150283		1.426	7316
3	STD2	3	ISONIAZI	2.668	151642		1.428	7288
4	STD2	4	ISONIAZI	2.667	150767		1.424	7576
5	STD2	5	ISONIAZI	2.672	152012		1.448	7392
Mean					151267			
%RSD					0.5			

Table no.7 Results of system suitability (RIFAMPICIN)

	Sample Name	Inj	Name	RT	Area	USPResolution	USPTailing	USPPlateCount
1	STD2	1	RIFAMPICI	3.170	2518297		1.420	7539
2	STD2	2	RIFAMPICI	3.172	2514902		1.407	7382
3	STD2	3	RIFAMPICI	3.173	2535682		1.402	7460
4	STD2	4	RIFAMPICI	3.170	2520334		1.428	7586
5	STD2	5	RIFAMPICI	3.174	2528250		1.390	7441
Mean					2523493			
%RSD					0.3			

Name: RIFAMPICI

SPECIFICITY

Specificity is the ability to assess unequivocally of an analyte in the presence of components which may be expected to be present. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

Blank, standard, placebo, all known related compounds, spiked sample, sample solutions were prepared and injected into the chromatographic system for identification and interference with the Isoniazid and Rifampicin peaks.

Placebo Interference:

A study to establish the interference of placebo was conducted. Sample preparation of placebo was done as that of test sample preparation of assay method. Chromatogram of placebo did not show any additional peaks. This indicated that the excipients used in the formulation did not interfere in the assay of Isoniazid and Rifampicin tablets. Resulted chromatograms were shown below.

Blank Interference:

A study to establish the interference of blank was conducted. Mobile phase was injected as per the test method and are shown below.

Fig no.5

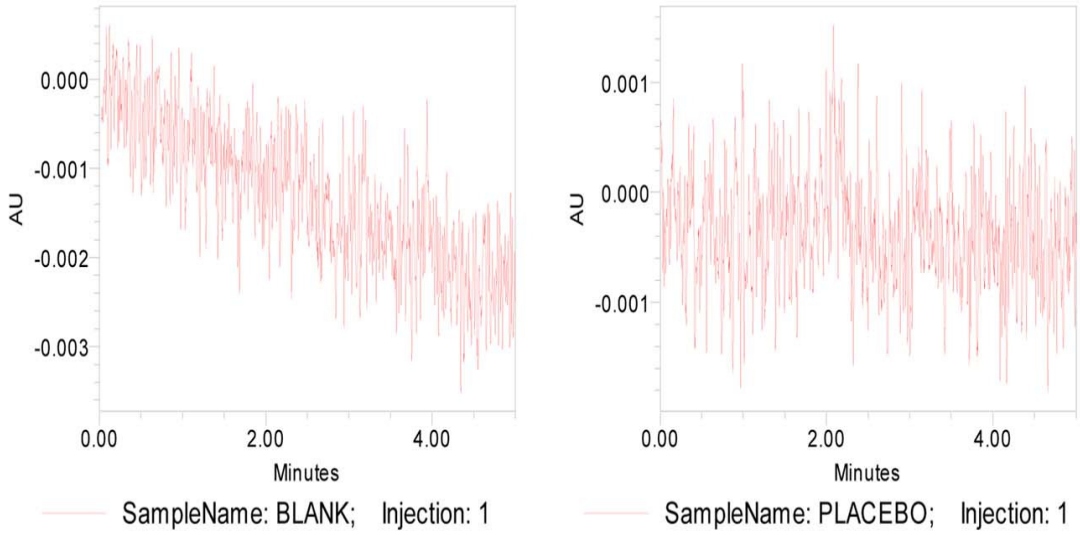


Table no.8 Component Summary Table for ISONIAZID

	Sample Name	Inj	Name	RT	Area
1	Blank	1	ISONIAZID	2.600	
2	Placebo	1	ISONIAZID	2.600	

Table no.9 Component Summary Table for RIFAMPICIN

	Sample Name	Inj	Name	RT	Area
1	Blank	1	RIFAMPICIN	3.100	
2	Placebo	1	RIFAMPICIN	3.100	

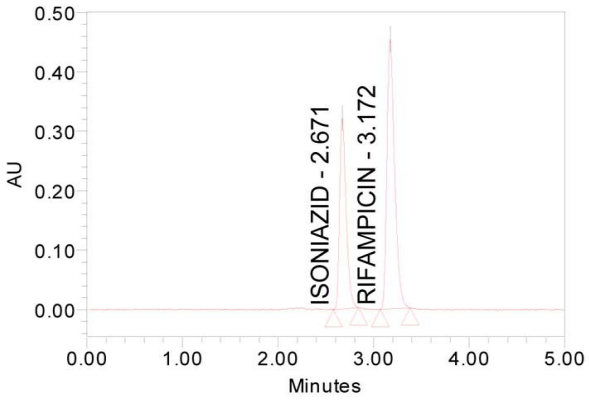
Precision:

Precision is the measure of the degree of repeatability of analytical method under normal operation and is normally expressed as %RSD for the statistically significant number of samples.

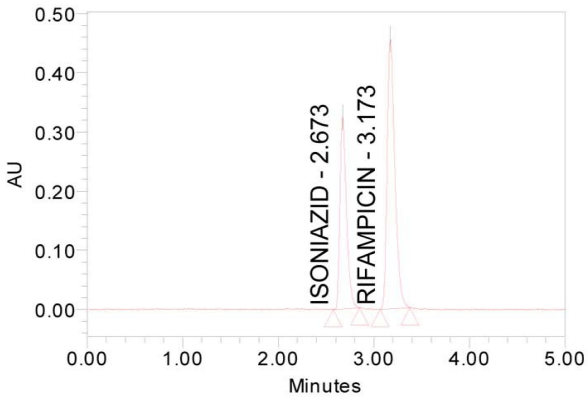
Method Precision:

Six sample preparations were prepared individually using single batch of Isoniazid and Rifampicin tablets (1/32 mg) as per test method and injected each solution. Resulted chromatogram was shown in the fig. no. 6. And data was shown in below table10.

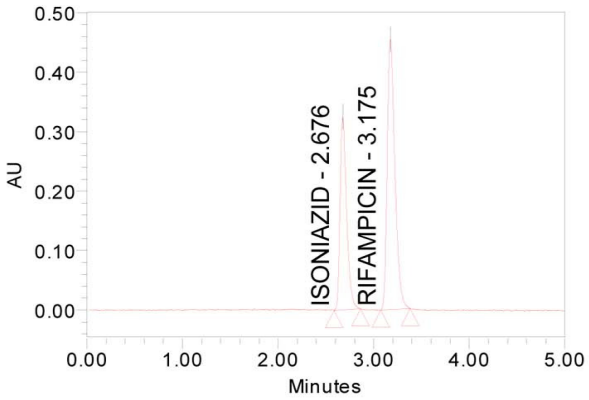
Fig no.6Chromatograms for sample



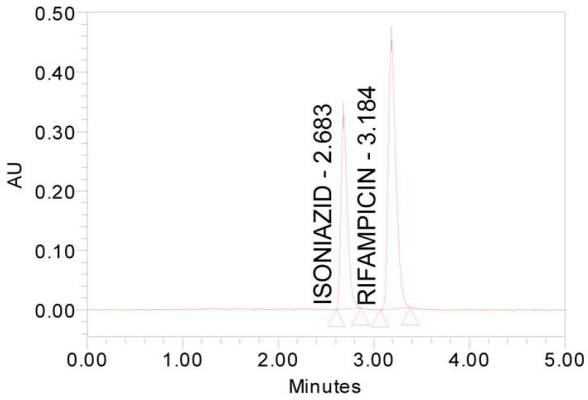
SampleName: PRECISION1; Injection: 1



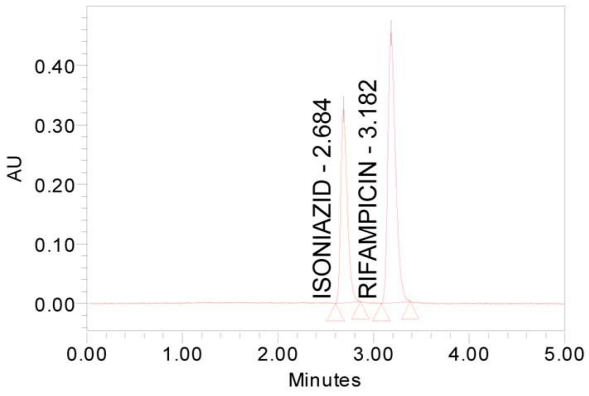
SampleName: PRECISION2; Injection: 1



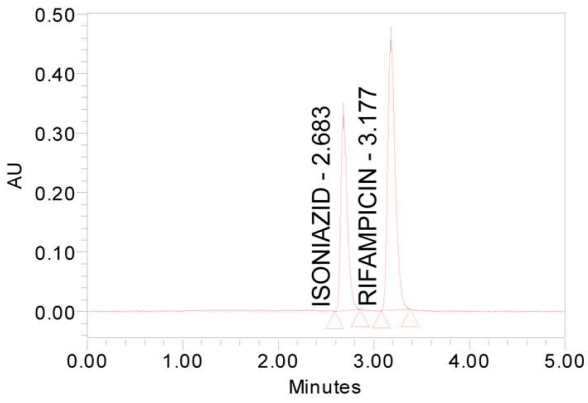
SampleName: PRECISION3; Injection: 1



SampleName: PRECISION4; Injection: 1



SampleName: PRECISION5; Injection: 1



SampleName: PRECISION6; Injection: 1

Table no.10 Data for system suitability (ISONIAZID)

Name: ISONIAZID

	SampleName	Inj	Name	RT	Area
1	PRECISION1	1	ISONIAZID	2.671	1519920
2	PRECISION2	1	ISONIAZID	2.673	1516120
3	PRECISION3	1	ISONIAZID	2.676	1511693
4	PRECISION4	1	ISONIAZID	2.683	1518392
5	PRECISION5	1	ISONIAZID	2.684	1514151
6	PRECISION6	1	ISONIAZID	2.683	1511595

Table no.11 Data for system suitability (RIFAMPICIN)

Name: RIFAMPICIN

	SampleName	Inj	Name	RT	Area
1	PRECISION1	1	RIFAMPICIN	3.172	2525898
2	PRECISION2	1	RIFAMPICIN	3.173	2527812
3	PRECISION3	1	RIFAMPICIN	3.175	2527001
4	PRECISION4	1	RIFAMPICIN	3.184	2529333
5	PRECISION5	1	RIFAMPICIN	3.182	2520997
6	PRECISION6	1	RIFAMPICIN	3.177	2521813

Table no.12 Calculated data for repeatability of Isoniazid and Rifampicin

S.No	Sample Weigh t	Sample Area-1	Sample Area-2	% Ass ay	% Assay
1	902.55	1519920	2525898	100	100
2	902.55	1516120	2527812	99	100
3	902.55	1511693	2527001	99	100
4	902.55	1518392	2529333	99	100
5	902.55	1514151	2520997	99	100
6	902.55	1511592	2521813	99	100
Average Assay				99	100
STD				0.23	0.13
%RSD				0.23	0.13

Acceptance criteria:

The % RSD of individual Isoniazid and Rifampicintablet from the six units should be not more than 2.0%.

All assay values should be within the 90.0 % - 110.0 % of label claim.

LINEARITY AND RANGE

Linearity

Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of an analyte in the sample.

Range

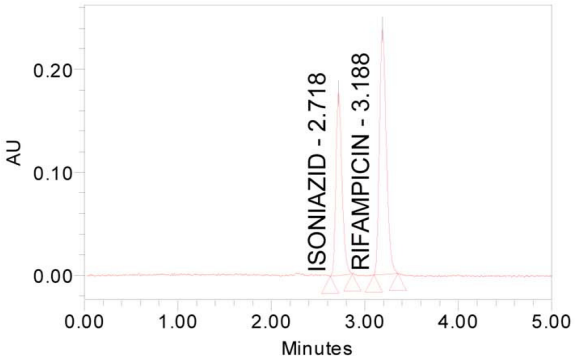
Range of an analytical procedure was the interval between the upper and lower concentration (amount) of an analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has suitable level of precision, accuracy and linearity.

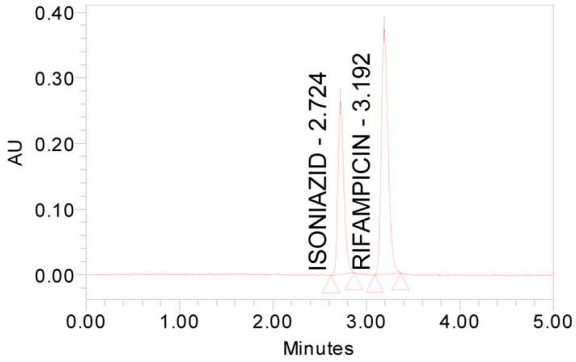
Standard solutions of Isoniazid and Rifampicin at concentration levels from 50 % to 150 % of standard solution were injected into HPLC system. The linearity graph was plotted from 50 % to 150

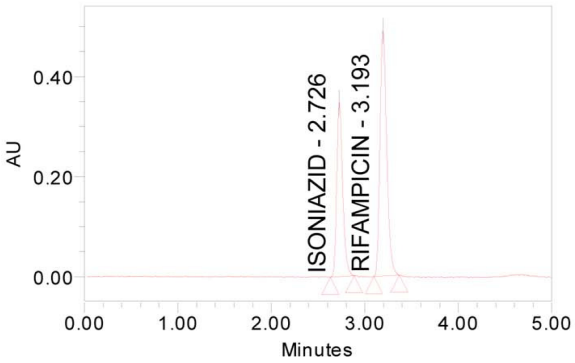
Acceptance criteria

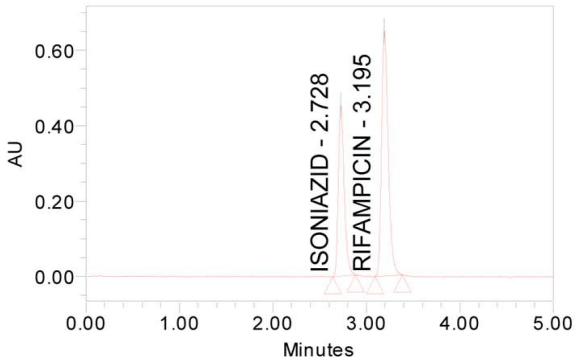
a. The correlation coefficient (r^2) must be NLT 0.999.
b. The RSD of replicate injections for lower and upper level concentrations should not be more than2.0 %.

Fig no.7Chromatograms for linearity









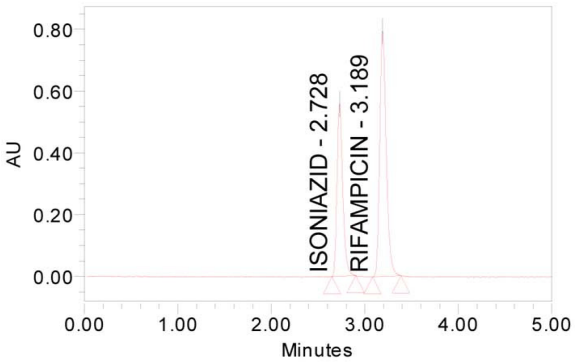


Table no.13 Data for system suitability (ISONIAZID)

Name: ISONIAZID

	SampleName	Inj	Name	RT	Area
1	LINEARITY-50%	1	ISONIAZID	2.718	756326
2	LINEARITY-75%	1	ISONIAZID	2.724	1136736
3	LINEARITY-100%	1	ISONIAZID	2.726	1518154
4	LINEARITY-125%	1	ISONIAZID	2.728	1895086
5	LINEARITY-150%	1	ISONIAZID	2.728	2266725

Fig no.8 Calibration curve for Isoniazid

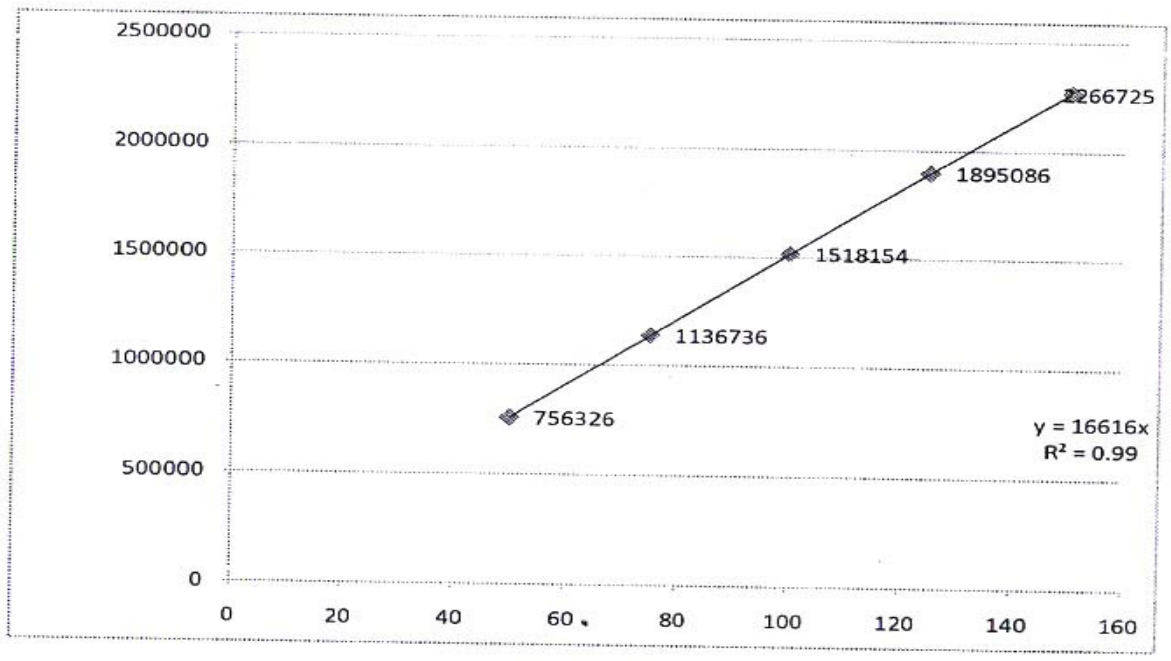


Table no.14 Calculated data for linearity (ISONIAZID)

ISONIAZID		
Conc%	Area	Concentration (µg/ml)
50	756326	600
75	1136736	900.00
100	1518154	1200.00
125	1895086	1500
150	2266725	1800

Table no.15 Data for system suitability (RIFAMPICIN)

Name: RIFAMPICIN

	SampleName	Inj	Name	RT	Area
1	LINEARITY-50%	1	RIFAMPICIN	3.188	1260352
2	LINEARITY-75%	1	RIFAMPICIN	3.192	1897143
3	LINEARITY-100%	1	RIFAMPICIN	3.193	2525789
4	LINEARITY-125%	1	RIFAMPICIN	3.195	3154260
5	LINEARITY-150%	1	RIFAMPICIN	3.189	3784968

Fig no.9 Calibration curve for Rifampicin

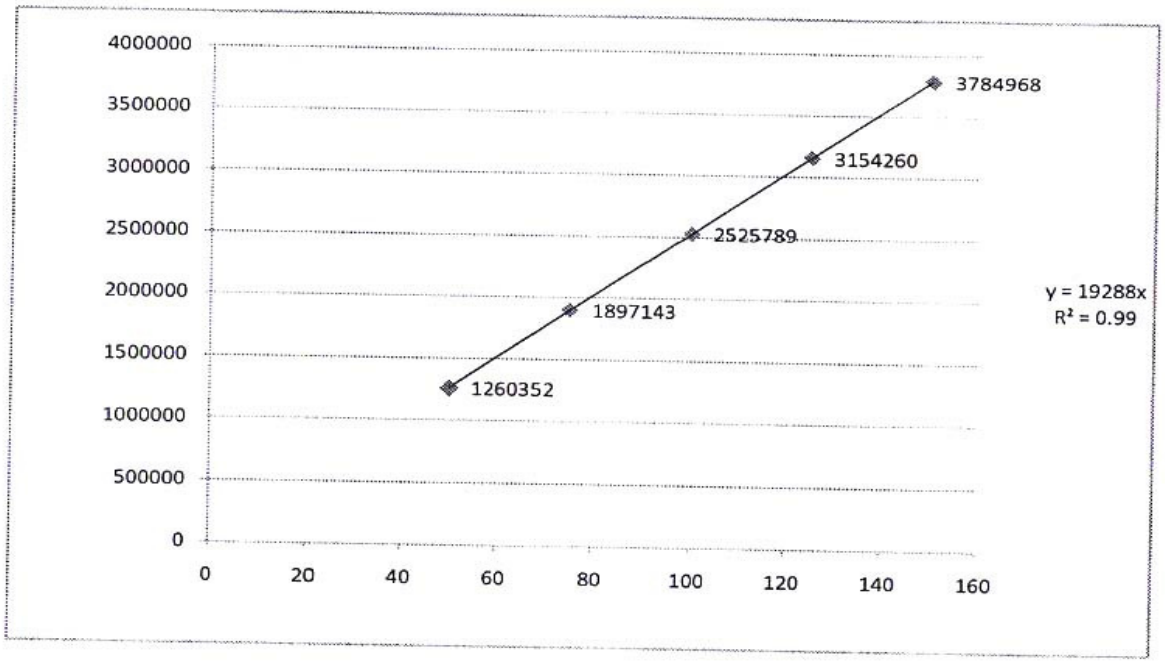


Table no.16 Calculated data for linearity (RIFAMPICIN)

RIFAMPICIN		
Conc%	Area	Concentration (µg/ml)
50	1260352	900
75	1897143	1350
100	2525789	1800
125	3154260	2250
150	3784968	2700.00

METHOD ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the values which is accepted either as a conventional true value or an accepted reference value for the observed value.

Fig no.10 Chromatograms for sample of 50% concentration

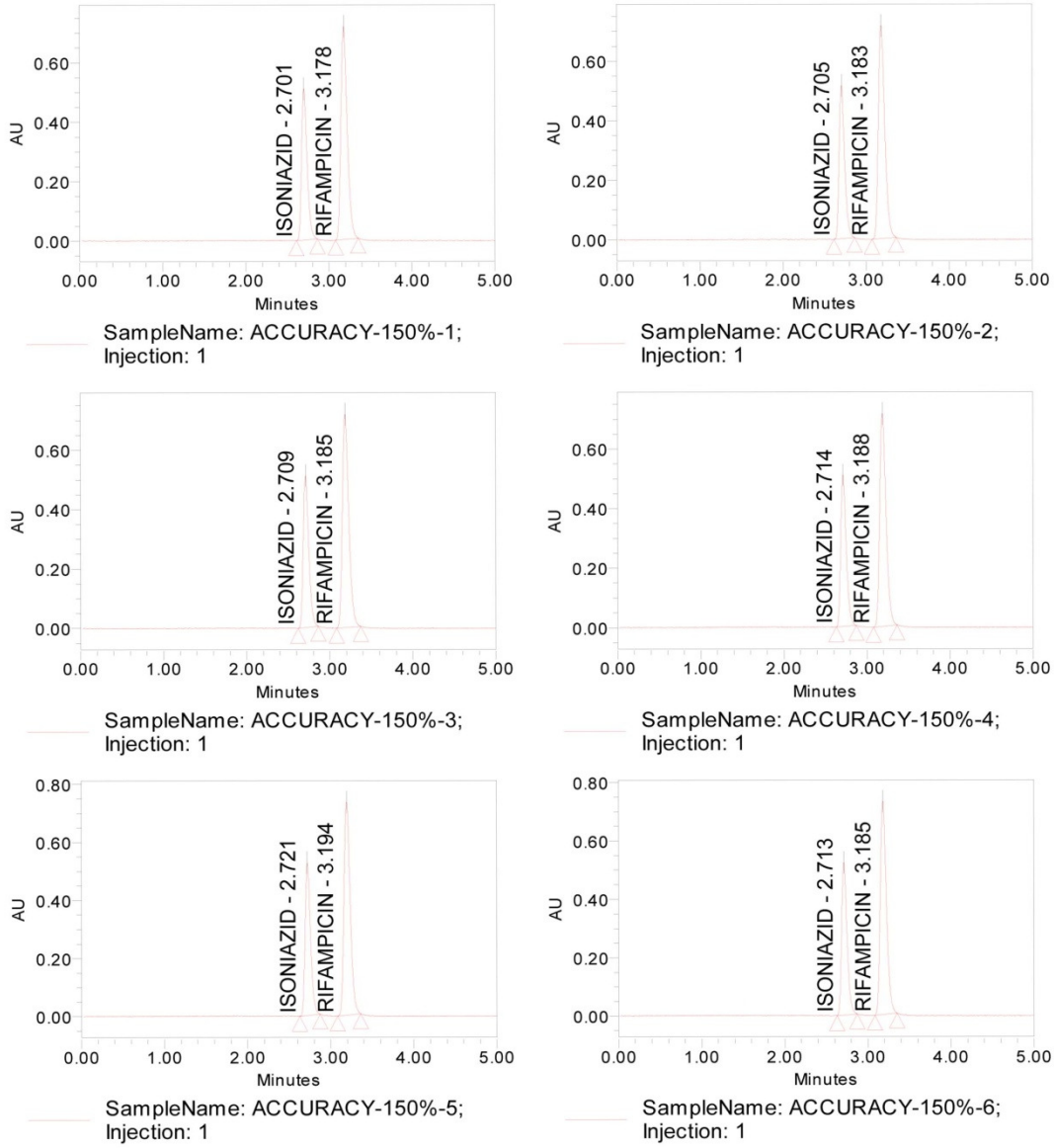


Table no.17 Data for accuracy of 50% concentration of Isoniazid

Name: ISONIAZID

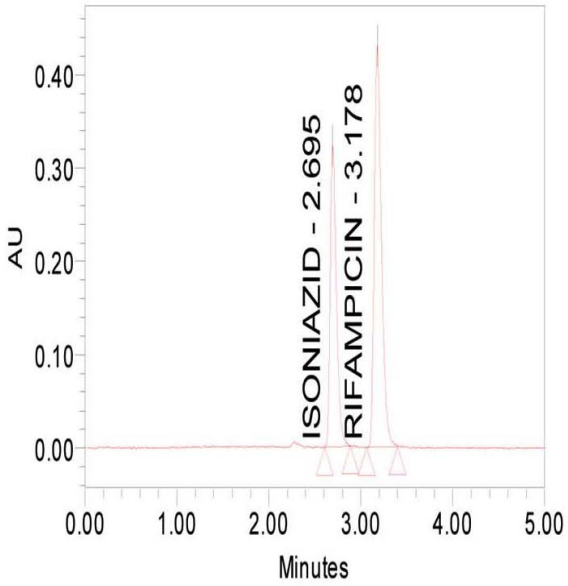
	SampleName	Inj	Name	RT	Area
1	ACCURACY-50%-1	1	ISONIAZID	2.688	756728
2	ACCURACY-50%-2	1	ISONIAZID	2.687	756907
3	ACCURACY-50%-3	1	ISONIAZID	2.687	756975
4	ACCURACY-50%-4	1	ISONIAZID	2.696	756326
5	ACCURACY-50%-5	1	ISONIAZID	2.699	756274
6	ACCURACY-50%-6	1	ISONIAZID	2.695	756141

Table no.18 Data for accuracy of 50% concentration of Rifampicin

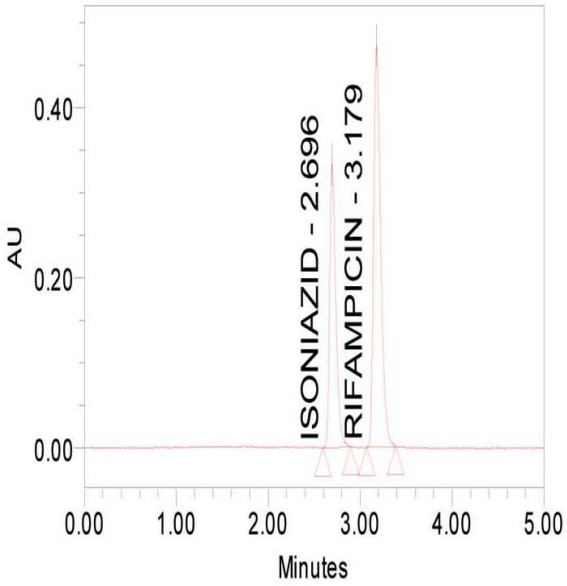
Name: RIFAMPICIN

	SampleName	Inj	Name	RT	Area
1	ACCURACY-50%-1	1	RIFAMPICIN	3.183	1265170
2	ACCURACY-50%-2	1	RIFAMPICIN	3.180	1262461
3	ACCURACY-50%-3	1	RIFAMPICIN	3.178	1261719
4	ACCURACY-50%-4	1	RIFAMPICIN	3.187	1263056
5	ACCURACY-50%-5	1	RIFAMPICIN	3.187	1268196
6	ACCURACY-50%-6	1	RIFAMPICIN	3.182	1262096

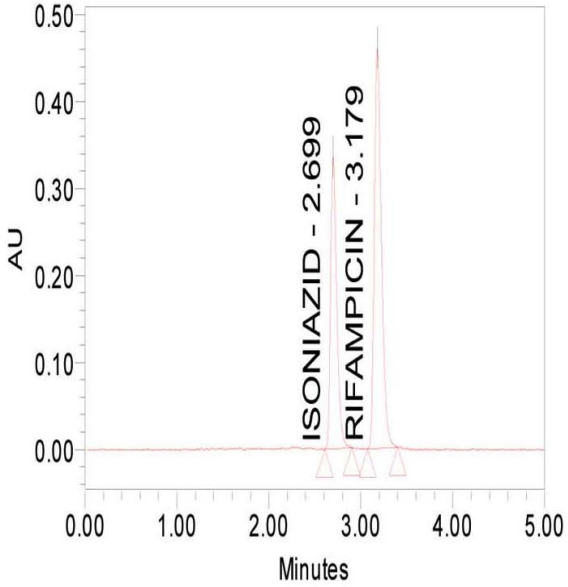
Fig no.11Chromatograms for sample of 100% concentration



SampleName: ACCURACY-100%-1;
Injection: 1



SampleName: ACCURACY-100%-2;
Injection: 1



SampleName: ACCURACY-100%-3;
Injection: 1

Table no.19 Data for accuracy of 100% concentration of Isoniazid

Name: ISONIAZID

	Sample Name	Inj	Name	RT	Area
1	ACCURACY – 100% -1	1	ISONIAZID	2.695	1512475
2	ACCURACY – 100% -2	1	ISONIAZID	2.696	1518251
3	ACCURACY – 100% -3	1	ISONIAZID	2.699	1512296

Table no.20 Data for accuracy of 100% concentration of Rifampicin

Name: RIFAMPICIN

	Sample Name	Inj	Name	RT	Area
1	ACCURACY – 100% -1	1	RIFAMPICIN	3.178	2523741
2	ACCURACY – 100% -2	1	RIFAMPICIN	3.179	2525279
3	ACCURACY – 100% -3	1	RIFAMPICIN	3.179	2528251

Fig no.12 Chromatograms for sample of 150% concentration

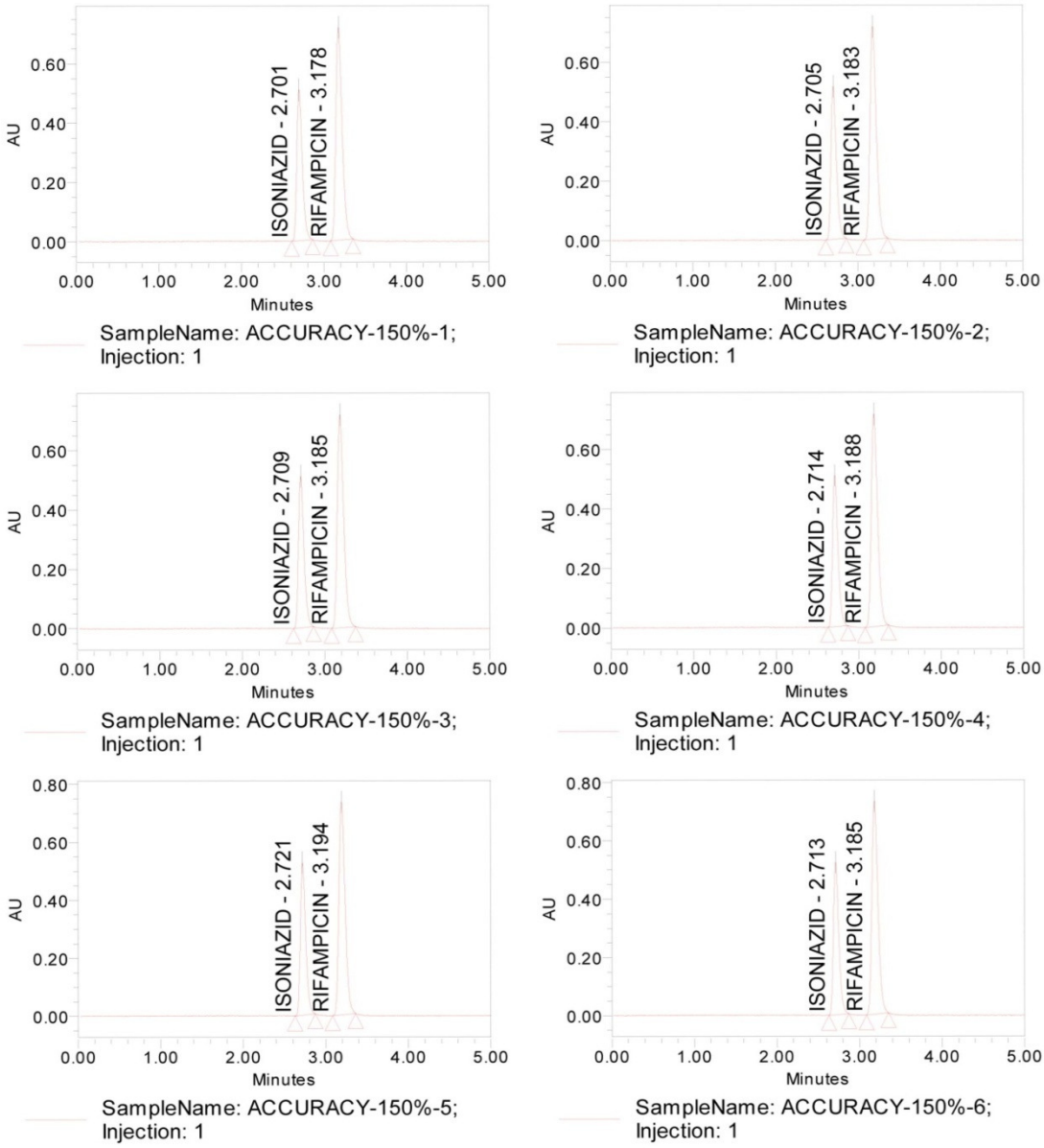


Table no.21 Data for accuracy of 150% concentration of Isoniazid

Name: ISONIAZID

	SampleName	Inj	Name	RT	Area
1	ACCURACY-150%-1	1	ISONIAZID	2.701	2260654
2	ACCURACY-150%-2	1	ISONIAZID	2.705	2262479
3	ACCURACY-150%-3	1	ISONIAZID	2.709	2260325
4	ACCURACY-150%-4	1	ISONIAZID	2.714	2262513
5	ACCURACY-150%-5	1	ISONIAZID	2.721	2263234
6	ACCURACY-150%-6	1	ISONIAZID	2.713	2266385

Table no.22 Data for accuracy of 150% concentration of Rifampicin

Name: RIFAMPICIN

	SampleName	Inj	Name	RT	Area
1	ACCURACY-150%-1	1	ISONIAZID	2.701	2260654
2	ACCURACY-150%-2	1	ISONIAZID	2.705	2262479
3	ACCURACY-150%-3	1	ISONIAZID	2.709	2260325
4	ACCURACY-150%-4	1	ISONIAZID	2.714	2262513
5	ACCURACY-150%-5	1	ISONIAZID	2.721	2263234
6	ACCURACY-150%-6	1	ISONIAZID	2.713	2266385

Table no.23 Results of Accuracy study (ISONIAZID)

ISONIAZID						
Spiked Level	**Sample Weight**	**Sample Area**	**µg/ml added**	**µg/ml found**	**% `Recovery**	**% Mean**
50 %	451.28	756728	594.007	594.91	100	100
50%	451.28	756907	594.007	594.05	100	
50%	451.28	756975	594.007	594.10	100	
50%	451.28	756326	594.007	594.59	100	
50%	451.28	756274	594.007	594.55	100	
100 %	902.55	1512475	1188.000	1189.04	100	100
100 %	902.55	1518251	1188.000	1193.58	100	
100 %	902.55	1512296	1188.000	1188.90	100	
150 %	1353.83	2260654	1782.007	1777.23	100	100
150 %	1353.83	2262479	1782.007	1778.66	100	
150 %	1353.83	2260325	1782.00	1776.9	100	

			7	7		
150 %	1353.83	2262513	1782.0007	1778.69	100	
150 %	1353.83	2263234	1782.0007	1779.26	100	
150 %	1353.83	2266385	1782.0007	1781.74	100	

Table no.24 Results of Accuracy study (RIFAMPICIN)

RIFAMPICIN				
Sample Area	**µg/ml added**	**µg/ml found**	**% Recovery**	**% Mean**
1265170	891.010	899.73	101	101
1262461	891.010	897.81	101	
1261719	891.010	897.28	101	
1263056	891.010	898.23	101	
1268196	891.010	901.89	101	
1262096	891.010	897.55	101	
2523741.00	1782.000	1795.78	101	101

2525279.0 0	1782.000	1795.87	101	
2528251.0 0	1782.000	1797.98	101	
3782160	2673.010	2689.71	101	101
3785672	2673.010	2692.21	101	
3782793	2673.010	269016	101	
3785575	2673.010	2692.14	101	
3788145	2673.010	2693.97	101	
3789803	2673.010	2695.15	101	

ROBUSTNESS

The robustness is a measure of method capacity to remain unaffected by small, deliberate variations in method parameters and provides an indication of method reliability during normal use.

Standard was prepared and injected into the chromatographic system as per the conditions specified in the method.

The same standard was reinjected by altering one parameter at a time, keeping other parameters constant.

A set of system suitability data was calculated for standards injected under altered method conditions and compared against the values generated under normal method conditions. The results

were tabulated in below table.

Acceptance Criteria

All the system suitability requirements must be met.

Method Parameters:

1. Flow Rate

(Normal flow is 1.0 ml/min)

a. Reduced flow ® 0.9 ml/min
b. Increased flow ® 1.1 ml/min

2. Column Operating Temperature

(Normal temperature is 30 °C)

a. Reduced Temperature ® 25 °C
b. Elevated Temperature ® 35 °C

Fig no.1 Chromatograms for sample flow and temperature

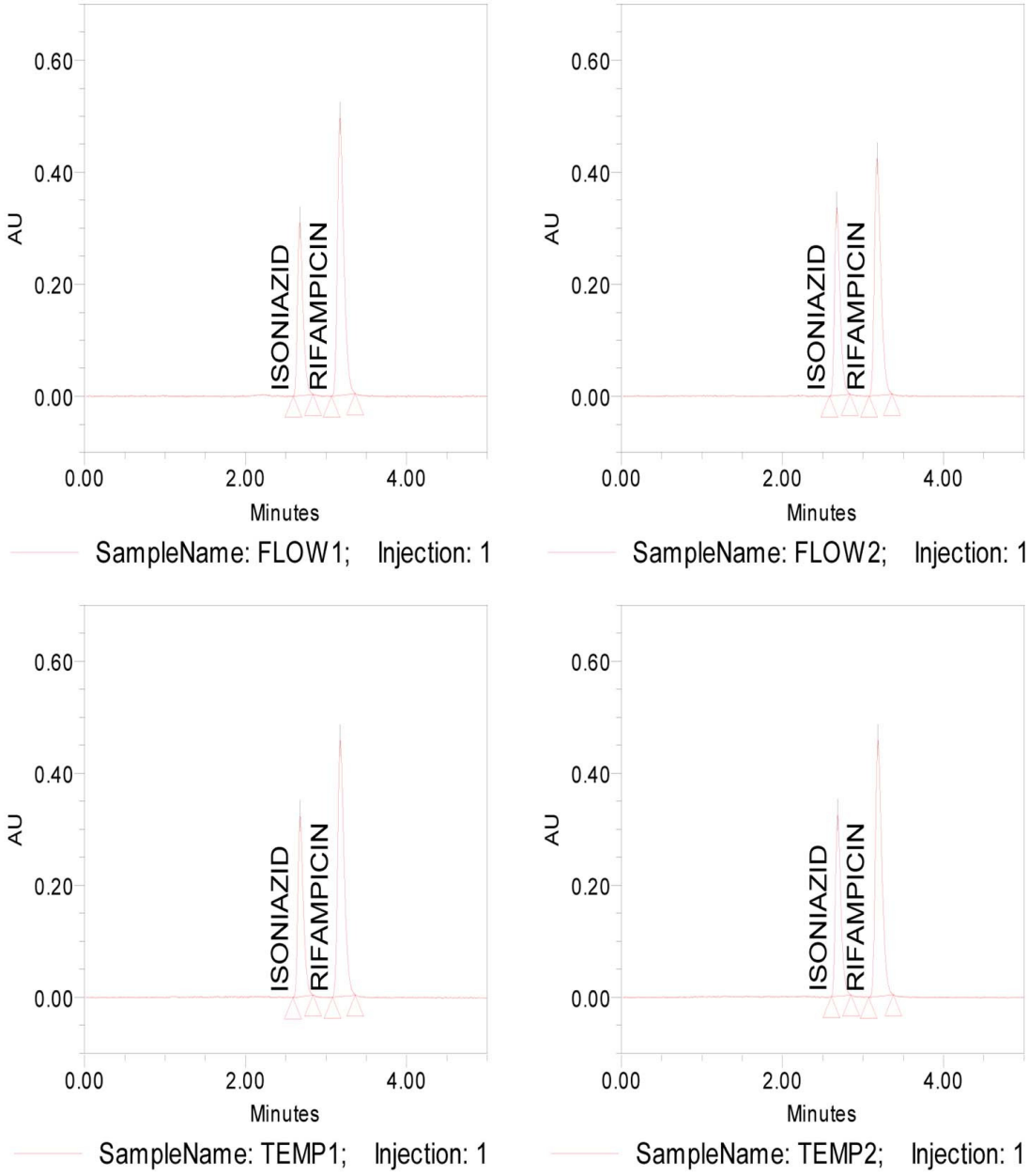


Table no.25 Data for variation in temperature and flow rate (ISONIAZID)

	SampleName	Inj	Name	RT	Area	USPResolution	USPTailing	USPPlateCount
1	FLOW1	1	ISONIAZID	2.673	14574		1.421	7399
2	FLOW2	1	ISONIAZID	2.675	15782		1.398	7247
3	TEMP1	1	ISONIAZID	2.677	15164		1.393	7510
4	TEMP2	1	ISONIAZID	2.685	15232		1.411	7515

Table no.26 Data for variation in temperature and flow rate (RIFAMPICIN)

	SampleName	Inj	Name	RT	Area	USPResolution	USPTailing	USPPlateCount
1	FLOW1	1	RIFAMPICIN	3.173	2748228		1.378	7386
2	FLOW2	1	RIFAMPICIN	3.173	2352295		1.410	7350
3	TEMP1	1	RIFAMPICIN	3.177	2544191		1.374	7233
4	TEMP2	1	RIFAMPICIN	3.186	2551703		1.364	7300

RESULTS AND DISCUSSION

Analytical method development and method validation was performed for RP-HPLC method for the Isoniazid and Rifampicin in tablet formulation as per ICH norms for the following parameters: system suitability, linearity and precision (repeatability), intermediate precision (ruggedness), specificity and accuracy. The summary of results obtained in analytical method development and validation were tabulated in table no.26.

VALIDATION SUMMARY REPORT

The observations and results obtained for each of the parameters like system suitability, linearity, precision (repeatability), specificity, accuracy and robustness lies well within the acceptance criteria. So the developed method was simple, specific, linear, precise, and accurate and robustness could be extensively used for the Isoniazid and Rifampicin in tablet formulation system.

Table no. 26Validation parameters and acceptance criteria for INH and RIF

S. No	Validation parameters	Specification	Results	
1	System suitability		Isoniazid	Rifampicin
	Retention time	Not applicable	2.660	3.172
	Tailing	NMT 2	1.469	1.412
	Resolution	NLT 2		3.697
	Theoretical plates	NLT 2500	7755	7613
	Similarity factor	0-98 to 1.02	0.99	0.99
	%RSD	NMT 2.0%	0.5	0.3
2	Specificity	There is no peak in blank at the Rt of analyte	Nil	Nil
		There is no peak in placebo at the Rt of analyte	Nil	Nil
3	Precision	The value should be between 97% to 103%	100	100
			99	100
			99	100
			99	100
			99	100
			99	100
		The %RSD of six replicate assay results NMT 2.0%	0.23	0.13
4	Accuracy (50%)	The value should be between 97% to 103%	100	101

	Accuracy (100%)	The value should be between 97% to 103%	100	101
	Accuracy (150%)	The value should be between 97% to 103%	100	101
5	Linearity	Correlation coefficient NLT 0.999	0.998	0.997
6	LOD	Not applicable	2.88 µg/ml	2.77 µg/ml
7	LOQ	Not applicable	9.58 µg/ml	9.22 µg/ml
8	Range	Not applicable	600µg to 1800 µg/ml	900µg to 2700 µg/ml
9	Robustness(Flow-1)			
	Tailing	NMT 2	1.421	1.378
	Resolution	NMT 2	Nil	3.596
	Theoretical plates	NLT 2500	7399	7386
	Robustness(Flow-2)			
	Tailing	NMT 2	1.398	1.410
	Resolution	NMT 2	Nil	3.578
	Theoretical plates	NLT 2500	7247	7350
	Robustness(Temp-1)			
	Tailing	NMT 2	1.393	1.374
	Resolution	NMT 2	Nil	3.590
	Theoretical plates	NLT 2500	7510	7233
	Robustness(Temp-2)			
	Tailing	NMT 2	1.411	1.364
	Resolution	NMT 2	Nil	3.601
	Theoretical plates	NLT 2500	7515	7300

CONCLUSION

From the results obtained, it was observed that the developed method was proven to be specific, precise, linear, accurate, rugged and robust and is suitable for its intended purpose. So the above work performed gives documented evidence that the analytical method for the Isoniazid and Rifampicin by RP-HPLC in tablet dosage forms will consistently analyze these drugs quantitatively and could be used for routine analysis.

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ABBREVATIONS

API	-	Active pharmaceutical ingredient
MeOH	-	Methanol
ACN	-	Acetonitrile
RT	-	Retention time
RH	-	Relative Humidity
CI	-	Confidence interval
NMT	-	Not more than
NLT	-	Not less than
NA	-	Not applicable
ND	-	Not detected
Fig	-	Figure
PDA	-	Photo diode array
P^{Ka}	-	Acid dissociation constant
K	-	Partition coefficient
%RSD	-	%Relative standard deviation
RPM	-	Revolutions per minute
ODS	-	Octa decyl silane
K'	-	Capacity factor
N	-	Theoretical plate number/efficiency
LOD	-	Limit of detection
LOQ	-	Limit of quantitation
ICH	-	International Conference on Hormonization
Conc	-	Concentration
Std	-	Standard
ppm	-	Parts per million

ppb	-	Parts per billion
DS	-	Drug substance
DP	-	Drug product
µL	-	Micro liter
µg	-	Micro gram
λ	-	Lambda
µ	-	Micron
mL	-	Milli liter
mg	-	Milli gram
nm	-	Nanometer
Max	-	Maximum
S.no	-	Serial number
^{o}C	-	Degree celsius
pH	-	Hydrogen ion concentration
HPLC	-	High performance liquid chromatography
UV	-	Ultraviolet-Visible